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SUPPLEMENTAL DECLARATION, POWER OF ATTORNEY, AND PETITION

As below named inventors, we hereby declare that: our residences, post office addresses and citizenships are as stated below next to our names; that we verily believe we are the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled "CONTROL OF GENE EXPRESSION USING A COMPLEX OF AN OLIGONUCLEOTIDE AND A REGULATORY PEPTIDE", the specification of which was filed on April 8, 2004, as application Serial No. 10/824,584.

We hereby state that we have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to in the Oath or Declaration.

We acknowledge the duty to disclose information which is material to patentability in accordance with Title 37, Code of Federal Regulations, Section 1.56.

We hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) on which priority is claimed:

Prior Foreign Application(s):

International Application No.: PCT/GB02/04633
International Filing Date: 11 October 2002
Priority Date Claimed: 11 October 2001
Entitled: CONTROL OF GENE EXPRESSION USING A COMPLEX OF AN OLIGONUCLEOTIDE AND A REGULATORY PEPTIDE

We hereby appoint NIKOLAI & MERSEREAU, P.A., (Customer Number 23595), a professional association, consisting of the following attorneys/agents and the following attorneys/agents individually: Thomas J. Nikolai, Registration No. 19,283; Charles G. Mersereau, Registration No. 26,205; and Steven E. Kahm, Registration No. 30,860; Mark A. Mersereau, Registration No. 46,926; and James J. Paige, Registration No. 50,886 of 900 Second Avenue South, Suite 820, Minneapolis, Minnesota 55402-3813; Telephone No. (612) 339-7461, our attorneys/agents with full power of substitution and revocation to prosecute this

application and to transact all business in the Patent and Trademark Office connected therewith.

Please direct all phone calls and correspondence to: C. G. Mersereau, Esq. at NIKOLAI & MERSEREAU, P.A., 900 Second Avenue South, Suite 820, Minneapolis, Minnesota 55402-3813; Telephone: (612) 339-7461.

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name: Stephen Hart	Name: Simak Ali
Date:	Date:
Residence: London, W12 0NN, UK	Residence: London, W12 0NN, UK
Citizenship: British	Citizenship: British
Post Office Address: Division of Medicine Cancer Cell Biology Section Imperial College School of Medicine Hammersmith Campus Room 601b Cyclotron Building Du Cane Road London W12 0NN United Kingdom	Post Office Address: Division of Medicine Cancer Cell Biology Section Imperial College School of Medicine Hammersmith Campus Room 601b Cyclotron Building Du Cane Road London W12 0NN United Kingdom

Name: Boris Tumi PuFong

Date:

Residence:
London, NW1 0TU, UK

Citizenship:
British

Post Office Address:
Gene Expression Technologies
The London BioScience
Innovation Centre
Royal College Street
London NW1 0TU
United Kingdom

Name: Andrew C. G. Porter

Date:

Residence:
London, W12 0NN, UK

Citizenship:
British

Post Office Address:
Gene Targeting Group
MRC Clinical Sciences Centre
Hammersmith Hospital
Du Cane Road
Hammersmith Campus
London W12 0NN
United Kingdom

Name: Laki Buluwela

Date:

Residence:
London, W12 0NN, UK

Citizenship:
British

Post Office Address:
Division of Medicine
Cancer Cell Biology Section
Imperial College School of
Medicine
Hammersmith Campus
Room 601b Cyclotron Building
Du Cane Road
London W12 0NN
United Kingdom

Name: Satu Vainikka

Date: 28/8/07

Residence:
London, N19 5RD, UK

Citizenship:
Finnish

Post Office Address:
Gene Expression Technologies
The London BioScience
Innovation Centre
Royal College Street
London NW1 0TU
United Kingdom

Name: John David Jenkinson

Date:

Residence:
London, NW1 0TU, UK

Citizenship:
British

Post Office Address:
Gene Expression Technologies
The London BioScience
Innovation Centre
Royal College Street
London NW1 0TU
United Kingdom

Name: Patrick Kanda

Date:

Residence:
London, NW1 0TU, UK

Citizenship:
United States of America

Post Office Address:
Gene Expression Technologies
The London BioScience
Innovation Centre
Royal College Street
London NW1 0TU
United Kingdom

ATTACHMENT "A"

Vol. 10, 4245s–4248s, June 15, 2004 (Suppl.)

Oblimersen Sodium (Genasense bcl-2 Antisense Oligonucleotide): A Rational Therapeutic to Enhance Apoptosis in Therapy of Lung Cancer

Roy S. Herbst[1] and Stanley R. Frankel[2,3]

[1]M. D. Anderson Cancer Center, Houston, Texas; [2]Genta Incorporated, Berkeley Heights, New Jersey; and [3]Greenebaum Cancer Center, University of Maryland at Baltimore, Baltimore, Maryland

ABSTRACT

Bcl-2 protein inhibits apoptosis and confers resistance to treatment with traditional cytotoxic chemotherapy, radiotherapy, and monoclonal antibodies. Oblimersen sodium is an antisense oligonucleotide compound designed to specifically bind to human *bcl-2* mRNA, resulting in catalytic degradation of *bcl-2* mRNA and subsequent decrease in bcl-2 protein translation. Both small cell and non-small cell lung cancer show baseline and inducible expression of bcl-2, which may contribute to resistance to therapy. Preclinical studies have shown that combining *bcl-2* antisense with chemotherapy improves antitumor response, increases apoptosis of tumor cells, and increases survival. Preliminary data from a large international randomized trial in melanoma show a trend toward increased survival and significantly improved response rates and response duration when oblimersen is added to dacarbazine. Phase I studies in small cell lung cancer patients demonstrate that oblimersen can be combined with paclitaxel or carboplatin and etoposide. The combination of docetaxel and oblimersen has been shown to be feasible in Phase I studies and is currently undergoing evaluation in comparison with docetaxel alone as first-line salvage therapy in patients refractory or relapsed after one prior chemotherapy regimen. Enhancement of the efficacy of anticancer treatments with oblimersen *bcl-2* antisense therapy represents a promising new apoptosis-modulating strategy.

BCL-2: A CRITICAL ANTIAPOPTOTIC PROTEIN

The development of cancer requires dysregulated proliferation combined with dysregulation of cell death. The components of the apoptotic program are therefore targets for anticancer therapy (1–4). The apoptotic control mechanisms of cells can be simplified to proapoptotic stimuli and antiapoptotic forces. Three families of apoptotic control proteins have been described based on their sequence homology, three-dimensional structure, and function: (*a*) proapoptotic proteins including Bax and Bak; (*b*) BH3 only proteins that are proapoptotic; and (*c*) antiapoptotic proteins including bcl-2 (2, 3, 5–8). The mechanisms of interaction between these classes of proteins continue to be described.

Located on the inner mitochondrial membrane, bcl-2 serves as a key inhibitor of apoptosis that blocks release of cytochrome *c* and maintains mitochondrial integrity (5, 8–10). By inhibiting apoptosis, bcl-2 confers resistance to treatment with traditional cytotoxic chemotherapy, radiotherapy, and monoclonal antibodies (11, 12). In studies of human cancer, most evidence suggests that bcl-2 contributes to a more malignant tumor phenotype. Elevated bcl-2 protein correlates with poor response to chemotherapy and/or hormonal therapy in non-Hodgkin's lymphoma, acute myelogenous leukemia, multiple myeloma, and prostate cancer (13–18). In xenograft models, nontumorigenic cell lines can be made highly tumorigenic by transfection with the *bcl-2* gene (14, 19, 20).

BCL-2 EXPRESSION IN NON-SMALL CELL LUNG CANCER (NSCLC)

Observations made in NSCLC suggest that the relationship between bcl-2 expression and tumor phenotype is complex and multifactorial. Most prior studies of bcl-2 expression in NSCLC have been conducted using immunohistochemistry on formalin-fixed, paraffin-embedded archival tissue. Contrary to the experience in hematological neoplasms and prostate cancer, early studies suggested that detection of bcl-2 in NSCLC by immunohistochemistry was associated with a lower risk of metastatic disease and possibly improved overall survival (21, 22). However, none of these early studies were correlated with the type of chemotherapy administered to patients. More recently, one study examined 34 tumor samples taken from patients with advanced NSCLC who were treated with the combination docetaxel and vinorelbine. In that study, 16% of cases were positive for bcl-2 expression, but there was no apparent correlation with response to therapy (23). In part, the small sample size of prior studies has limited proper statistical analysis. Unexamined variables of potential importance include the relative expression of other genes, stage of disease, source of biopsy specimen from a primary tumor or metastatic site, and whether the biopsy is from a previously treated or chemotherapy-naive patient. In lung cancer cell lines, *bcl-2* antisense reduced bcl-2 protein expression, enhancing apoptotic activity of standard anticancer drugs (24–26).

The medical literature is inconsistent regarding the importance of bcl-2 alone as a clinical prognostic factor. Various methods (*e.g.*, immunohistochemistry or Northern or Western analysis) have yielded nonuniform results. Other problems have

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Requests for reprints: Roy S. Herbst, Thoracic Medical Oncology, M. D. Anderson Cancer Center, The University of Texas, 1515 Holcombe Boulevard-432, Houston, TX 77030-4009. Phone: (713) 792-6363; Fax: (713) 796-8655; E-mail: rherbst@mdanderson.org.

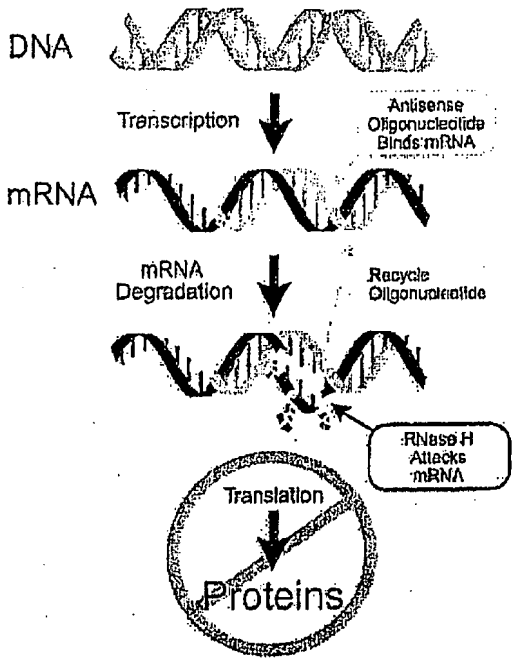

Fig. 1 Schematic representation of the binding of oblimersen to bcl-2 mRNA, resulting in decreased bcl-2 protein translation.

included contamination of solid tumor biopsies with epithelial, blood, and stromal cells and a generally low standard for quality control, particularly with regard to basal levels of expression and diurnal variation (27). More recent studies have assessed relative imbalances in anti- *versus* proapoptotic protein pools, some of which suggest that a high ratio of bcl-2:Bax may be clinically more informative. Overall, few firm conclusions can be drawn from these studies.

ANTISENSE THERAPY TO TARGET BCL-2

Oblimersen sodium (G3139, Genasense; Genta Inc., Berkeley Heights, NJ) is an antisense phosphorothioate oligonucleotide compound designed to specifically bind to the first six codons of the human bcl-2 mRNA sequence, resulting in degradation of *bcl-2* mRNA and subsequent decrease in bcl-2 protein translation and intracellular concentration (Fig. 1; Refs. 28 and 29). Oblimersen is the first oligonucleotide to demonstrate proof of principle of an antisense effect in human tumors by the documented down-regulation of the target bcl-2 protein (30).

PRECLINICAL ACTIVITY OF OBLIMERSEN

A growing body of preclinical and clinical evidence suggests that oblimersen synergizes with many cytotoxic and biological/immunotherapeutic agents against a variety of hematological malignancies and solid tumors. Studies of oblimersen in xenograft models have shown marked enhancement of the efficacy of standard cytotoxic chemotherapy and of rituximab in several cancers including non-Hodgkin's lymphoma, melanoma, breast cancer, gastric cancer, and NSCLC (Table 1; Ref. 31). Durable regressions of aggressive human breast cancer xenografts have been observed after combination therapy with docetaxel and oblimersen (32). Oblimersen down-regulates *bcl-2* mRNA within 48 h and protein levels within 96 h in *ex vivo*-treated myeloma cells (33). This down-regulation is associated in a sequence-specific manner with sensitization of myeloma cells to cytotoxic activity of dexamethasone and doxorubicin (33, 34).

CLINICAL STUDIES OF OBLIMERSEN

Phase I/II trials indicate that oblimersen provides biologically relevant plasma levels, down-regulates target bcl-2 protein within 3–5 days of initiating treatment, and yields an acceptable safety profile. The most common toxicities are low-grade fever that is usually self-limiting and fatigue, particularly with longer durations of infusion (35, 36).

In a small pilot study, 12 patients with chemorefractory small cell lung cancer (SCLC) received paclitaxel (150 mg/m^2 on day 6 of a 21-day cycle) plus oblimersen [3 mg/kg on days 1–8 of the cycle (36)]. All had previous treatment with etoposide plus a platinum; five patients had been treated with 1–3 additional chemotherapy regimens, and four patients had progressed after prior paclitaxel treatment. No objective responses occurred on the combination paclitaxel/oblimersen regimen; four patients had stable disease after 2 treatment cycles, with two of the four patients progressing within 1 month and terminating therapy with cycle 3. Of the two remaining patients, one maintained stable disease until cycle 6, and one remained stable over 10 cycles of therapy and free of progression for over 1 year. It is of interest that only the patient with prolonged stable disease had consistently high plasma oblimersen levels.

Given the very poor prognosis for chemorefractory SCLC, these findings were sufficiently promising to prompt a dose-finding study in 16 previously untreated SCLC patients (37). Patients were divided into three cohorts to receive either of the following regimens: (*a*) 5 mg/kg oblimersen on days 1–8 of a

Table 1 Oblimersen sodium in NSCLC[a] human H-460 cell xenografts

	Oblimersen [mg/k/day (s.c. days 8–14, bid)]	Docetaxel [mg/k/day (i.v., days 9, 12, and 15)]	Drug deaths	(T−C) days	Log cell kill	Complete response
Oblimersen alone	5		0/7	5.0	0.8	0/7
Docetaxel alone		20.3	0/7	13.2	2.0	0/7
Combination therapy	5	20.8	0/7	20.9	3.1	4/7

[a] NSCLC, non-small cell lung cancer; bid, twice a day; T−C, tumor growth delay.

21-day cycle, carboplatin at a dose of area under the curve 6 on day 6, and 80 mg/m^2 etoposide on days 1–8 of cycle; (*b*) 5 mg/kg oblimersen on days 1–8 of a 21-day cycle, carboplatin at a dose of area under the curve 5 on day 6, and 80 mg/m^2 etoposide on days 1–8 of cycle; or (*c*) 7 mg/kg oblimersen on days 1–8 of a 21-day cycle, carboplatin at a dose of area under the curve 5 on day 6, and 80 mg/m^2 etoposide on days 1–8 of cycle. Of 14 evaluable patients, partial response was seen in 12 patients, and stable disease was seen in 2 patients. Dose-limiting toxicity (grade 4 neutropenia) occurred in two of three evaluable patients in cohort 1, but these patients were able to continue with a dose reduction of the carboplatin to area under the curve 5; one grade 3 neutropenia occurred in cohort 2; in cohort 3, five of six evaluable patients experienced at least one episode of neutropenia (grade 3 or 4), and four of six patients experienced thrombocytopenia (grade 3), with toxicity observed primarily in later cycles. The majority of patients in all three cohorts were able to complete all six planned cycles of therapy.

The dose of 7 mg/kg/day oblimersen is currently being studied in combination with carboplatin and etoposide as front-line therapy in 50 patients with SCLC in a Phase II trial by the Cancer and Leukemia Group B (CALGB 30103 trial). Oblimersen is also being evaluated in conjunction with docetaxel as second-line therapy in relapsed or refractory stage IIIB-IV NSCLC. This is a multicenter randomized trial in which patients will be randomized to docetaxel (75 mg/m^2 on day 5) alone or docetaxel plus oblimersen (7 mg/kg on days 1–8) for up to eight 21-day cycles. The primary end point will be survival, with tumor response and time to progression as secondary end points (Fig. 2).

Phase I/II studies of oblimersen have also been undertaken in melanoma (33, 38), prostate cancer (39–41), and refractory acute leukemias (42). Dose-limiting toxicity of thrombocytopenia was reached at 12 mg/kg/day for 5 days when combined with decarbazine 1000 mg/m^2. Samples from tumors in these studies have shown decreases in bcl-2 protein after oblimersen therapy. Randomized clinical trials are currently under way to evaluate the efficacy and tolerability of oblimersen in combination with cytotoxic chemotherapy in lung cancer, chronic lymphocytic leukemia, multiple myeloma, and malignant melanoma. In addition, nonrandomized trials are under way to evaluate oblimersen in combination with different classes of chemotherapy agents and monoclonal antibodies in gastric, colon, breast, hepatocellular, prostate, and Merkel cell cancers; non-Hodgkin's lymphoma; acute myeloid leukemia; chronic myelogenous leukemia; and multiple myeloma.

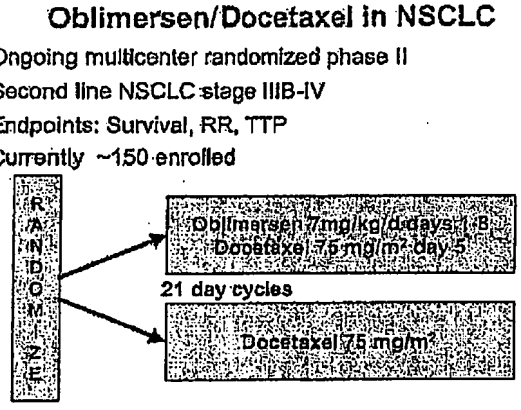

Fig. 2 Design of ongoing multicenter randomized trial of oblimersen sodium and docetaxel as second-line therapy in relapsed or refractory stage IIIB-IV non-small cell lung cancer.

OPEN DISCUSSION

Dr. Mark Socinski: In the second-line trials of docetaxel plus or minus oblimersen, what are the statistical assumptions now with that number of patients? Was survival the primary end point?

Dr. Roy S. Herbst: Yes, it was based on survival.

Dr. Raymond DuBois: With this particular pathway, there are a lot of other antiapoptotic members such as MCL1. Have you seen any change in their expression when you knocked down bcl-2?

Dr. Herbst: We have not looked at that because we have been doing only a clinical study. Preclinically, that's something that needs to be investigated: there are a number of different mechanisms and different members of this pathway. Some of the small molecules that are available now might also come into play.

Dr. Ramaswamy Govindan: That was going to be my question. If other signals can compensate, then whatever you knock out may not make a big difference. Given that this inhibitor is very specific, the initial signals are not that stellar.

Dr. Herbst: My take would be the following: we have a drug that we know is pharmacologically deliverable. We know that it's safe. We have some evidence in a large trial that it actually works. I think this is now our chance in lung cancer to do one of two things. We can take this to a big Phase III trial, and maybe we need to do that, but we should also consider a small 30-patient biological trial and actually collect tissue and blood monocytes and ask if we are hitting the target and if we are seeing activity as a single agent. Perhaps now is the time to ask more scientific questions.

Dr. Geoffrey Shapiro: In SCLC, is bcl-2 there *de novo*, or is it only there when resistance develops? Because if it's there *de novo*, then one might argue that it is not really preventing cell death because we have very good response rates upfront.

Dr. Herbst: So perhaps we can then improve on an already good response rate or help with resistant disease? Again, there are all these unknowns that need to be addressed.

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ATTACHMENT "B"

ANTISENSE & NUCLEIC ACID DRUG DEVELOPMENT 12:193–213 (2002)

Review

Oblimersen Bcl-2 Antisense: Facilitating Apoptosis in Anticancer Treatment

RICHARD J. KLASA,[1] AMANDA M. GILLUM,[2] ROBERT E. KLEM,[2] and STANLEY R. FRANKEL[2]

ABSTRACT

The components of the apoptotic program are targets for anticancer therapy. Bcl-2 protein inhibits apoptosis and confers resistance to treatment with traditional cytotoxic chemotherapy, radiotherapy, and monoclonal antibodies (mAb). Oblimersen sodium (G3139, Genasense™, Genta Inc., Berkeley Heights, NJ) is an antisense oligonucleotide (AS-ON) compound designed to specifically bind to the first 6 codons of the human *bcl-2* mRNA sequence, resulting in degradation of *bcl-2* mRNA and subsequent decrease in Bcl-2 protein translation. Oblimersen is the first oligonucleotide to demonstrate proof of principle of an antisense effect in human tumors by the documented downregulation of the target Bcl-2 protein. A growing body of preclinical and clinical evidence suggests that oblimersen synergizes with many cytotoxic and biologic/immunotherapeutic agents against a variety of hematologic malignancies and solid tumors. Randomized clinical trials are currently underway to evaluate the efficacy and tolerability of oblimersen in combination with cytotoxic chemotherapy in chronic lymphocytic leukemia, multiple myeloma, malignant melanoma, and non-small cell lung cancer. In addition, nonrandomized trials are under way to evaluate oblimersen in non-Hodgkin's lymphoma, acute myeloid leukemia, and hormone-refractory prostate cancer. Preclinical data also support the clinical evaluation of oblimersen in additional tumor types, including chronic myelogenous leukemia and breast, small cell lung, gastric, colon, bladder, and Merkel cell cancers. Enhancement of the efficacy of anticancer treatments with oblimersen Bcl-2 antisense therapy represents a promising new apoptosis-modulating strategy, and ongoing clinical trials will test this therapeutic approach.

APOPTOSIS AND CANCER

APOPTOSIS, OR PROGRAMMED CELL DEATH, is a critical and complex process in tissue homeostasis (reviewed by Reed, 1999, 2000; Yang and Korsmeyer, 1996). In the average human adult, it is responsible for the death of tens of billions of cells daily. Self-renewing tissues, including the skin, gut, and bone marrow, depend on this process to accommodate a similar number of newly created cells (Reed, 1999). Apoptosis culminates in the fragmentation of the cell into membrane-encased bodies that are cleared by phagocytosis without leading to inflammatory reaction or tissue scarring. Active inhibition of apoptosis allows a cell to live longer, which may be desirable for nonrenewing tissues (neurons) or lymphoid cells important to immune memory function. Antiapoptotic processes contribute to neoplasia by allowing an environment that permits genetic instability and mutations and that evades cell cycle checkpoints that normally induce apoptosis. Neoplasia, therefore, seems to result not only from unrestrained cell proliferation but also from insufficient apoptotic turnover, leading to overexpansion of a cell population (Nicholson, 2000). Further, this environment promotes survival during metastasis despite decreased availability of oxygen and nutrients and confers resistance to other important stresses, such as cytotoxic chemotherapy, antitumor antibodies, and radiation therapy. Consequently, targeted modulation of apoptosis has become an intensely investigated strategy for the development of new treatments for cancer (Nicholson, 2000).

[1]Division of Medical Oncology and Department of Advanced Therapeutics, British Columbia Cancer Agency, Vancouver, British Columbia V5Z 4E6, Canada.
[2]Genta Inc., Berkeley Heights, NJ 07922.

Bcl-2: A KEY REGULATOR OF APOPTOSIS AND LOGICAL ANTICANCER TARGET

The fundamental steps of apoptosis are primarily regulated by three molecular families: the Bcl-2 family, the tumor necrosis factor (TNF) family, and the caspases (Reed, 1999, 2000; Korsmeyer, 1999). The Bcl-2 family of proteins regulates the mitochondria-mediated, or intrinsic, apoptosis pathway (Fig. 1). Various cell death stimuli can cause mitochondrial membrane changes, resulting in the release of caspase-activating factors, such as cytochrome c, into the cytosol. Once in the cytosol, cytochrome c complexes to and activates apoptotic protease activating factor-1 (Apaf-1), which begins the activation of a cascade of caspase proteases. These proteases are mediators of cell death. The Bcl-2 protein works in a manner as yet incompletely characterized to block pores or otherwise stabilize the mitochondrial membrane such that cytochrome c is not released even when stimuli are present, thus blocking initiation of the intrinsic apoptosis pathway.

The Bcl-2 family includes both apoptosis-suppressing (e.g., Bcl-2) and apoptosis-inducing (e.g., Bax) proteins. Alteration in the expression of either type occurs frequently in many human cancers. Although a variety of genetic mechanisms can cause gain or loss of expression of Bcl-2 family proteins, the final common pathway leads to an alteration in the sensitivity of a cell to engage the apoptotic program through caspase activation (Fig. 1).

Overexpression or imbalance of Bcl-2 is associated with tumor cell resistance to a host of apoptotic stimuli, including chemotherapeutic agents, antitumor antibodies, radiation, hypoxia, cell detachment from extracellular matrix (ECM), growth factor withdrawal, and increases in cytosolic calcium (Reed, 1999). The level of Bcl-2 expression also may have implications for novel cancer therapy strategies, such as angiogenesis inhibition, suppression of tumor cell invasion and metastasis, and blockade of growth factor receptors (anti-human epidermal growth factor receptor 2 [HER2], anti-epidermal growth factor receptor [EGFR]).

The fact that Bcl-2 impedes the apoptotic response normally induced by chemotherapeutic agents further defines it as a multidrug-resistance protein (Reed, 1999). Aberrant Bcl-2 expression does not interfere with cytotoxic drug entry or accumulation in tumor cells or with the initial degree of damage or rate of cellular repair but rather prevents triggering of the cell death cascade. Tumor cells may thus experience drug-induced cell cycle inhibition but remain viable for extended periods (Gross et al., 1999). The prevention of apoptosis by Bcl-2 essentially converts cytotoxic anticancer drugs to cytostatic agents, potentially resulting in lower response rates to chemotherapeutic agents, earlier relapse, and shortened patient survival (Reed, 1999). Expression of Bcl-2 appears to confer a clinically relevant chemoresistant phenotype on many types of cancer cells, including non-Hodgkin's lymphoma (NHL) (Schmitt et al., 2000; Reed et al., 1994), acute myelogenous leukemia (AML) (Karakas et al., 1998), chronic lymphocytic leukemia (CLL) (Pepper et al., 1996; Hanada et al., 1993; Schena et al., 1992; Lazaridou et al., 2000), multiple myeloma (MM) (Tian et al., 1996; Hu and Gazitt, 1996; Gazitt et al., 1998), melanoma (Selzer et al., 1998; Grover and Wilson, 1996; Cerroni et al., 1995), prostate cancer (Gleave et al., 1999; Scott et al., 2001; Sullivan et al., 1998), breast cancer (Lanzafame et al., 1998), colorectal cancer (Ochoa et al., 2001a,b; H.-B. Yang et al., 1999), and small cell and non-small cell lung cancer (SCLC and NSCLC) (Jiang et al., 1995) (Fig. 2).

bcl-2 knockout mice (mice in whom the *bcl-2* gene has been eliminated) demonstrate the role of Bcl-2 in normal melanocyte and lymphocyte development (Yamamura et al., 1996). These mice are viable but experience a rapid degeneration of hair bulb melanocytes after birth (Yamamura et al., 1996; Kamada et al., 1995; Veis et al., 1993; Nakayama et al., 1994) and are severely lymphopenic (Kamada et al., 1995; Veis et al., 1993; Nakayama et al., 1994). In contrast, *bcl-2* transgenic mice that constitutively overexpress Bcl-2 develop B cell malignancies, demonstrating that aberrant expression of Bcl-2 has a role in the development of B cell lymphomas (Veis et al., 1993; McDonnell and Korsmeyer, 1991).

ANTISENSE OLIGONUCLEOTIDES

Reverse complementary or antisense oligonucleotides (AS-ONs) are short sequences of single-stranded deoxyribonucleotides complementary to gene coding regions that are designed to hybridize by Watson-Crick base pairing to messenger RNA (mRNA) sequences, making degradation possible (Zamecnik and Stephenson, 1978; Stephenson and Zamecnik, 1978). Naturally occurring antisense sequences have been identified that control gene expression in a variety of systems, allowing for possible therapeutic development (Mizuno et al., 1984; Izant and Weintraub, 1984). The formation of a heteroduplex of mRNA with the DNA of the AS-ON engages RNase H. This enzyme cleaves the mRNA moiety, destroying the message, and in theory releases an intact therapeutic AS-ON molecule to catalytically hybridize to another mRNA sequence (Winterberger, 1990). The resulting decrease in the target mRNA pool leads to reduction in the specific encoded protein (Fig. 3). The AS-ON also may prevent the mRNA from appropriately docking with the ribosomal machinery, thereby stopping translation into a functional protein and expression of that protein in the cell.

AS-ONs of 16–24 bases in length allow target specificity, whereas shorter or longer sequences may lead to random hybridizations. Selection of target areas within a mRNA must consider its tertiary structure, which will determine the accessibility of an area for hybridization. Target areas are identified empirically by screening series of oligonucleotides for the ability to decrease target mRNA levels. Oligonucleotide library screening also has identified RNA sites that are most accessible to hybridization and correlated these sites with protein down-regulation and biologic function (Ho et al., 1996, 1998). The first 6 codons of the open reading frame (ORF) at the AUG start site frequently have demonstrated accessibility to hybridization and have been chosen for initial AS-ON development against an assortment of genes.

The correlation of a biologic effect with the specific down-regulation of target mRNA and protein *in vivo* has been a primary focal point of AS-ON research. AS-ONs also can be potent immunostimulators if the sequence has unmethylated CpG motifs coupled with certain flanking sequences (Krieg et al., 1995). Thus, therapeutic activity could result in part from non-specific systemic immune effects rather than a specific AS-ON-

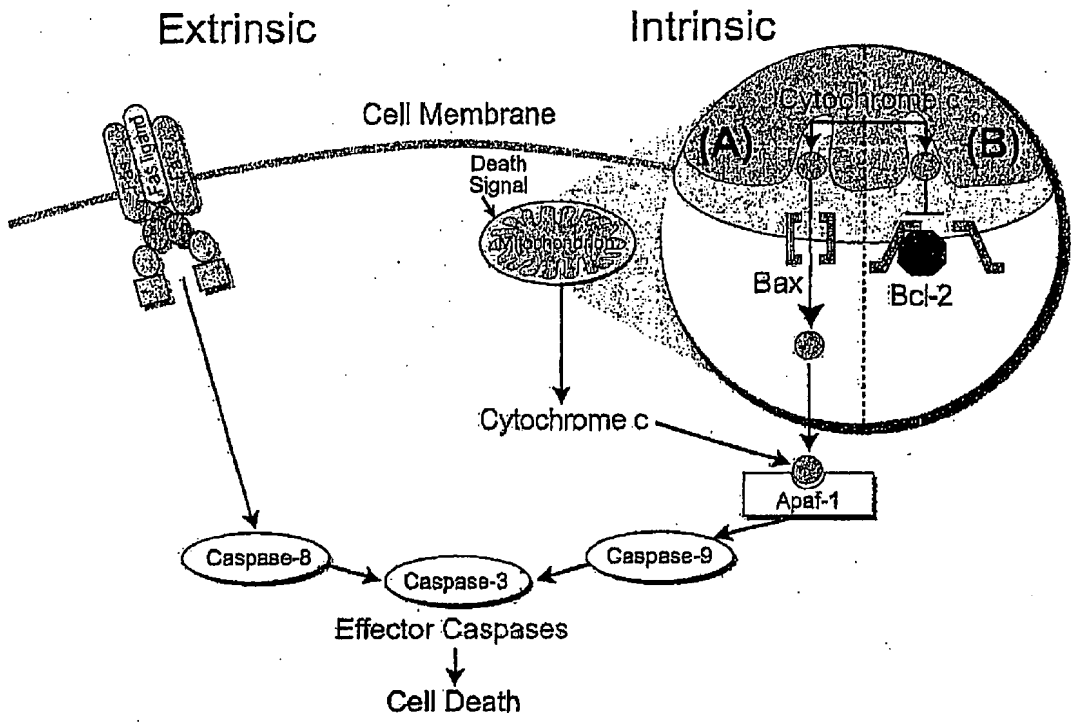

FIG. 1. Simplified schematic of extrinsic (left) and intrinsic (right) pathways of apoptosis. Both pathways converge by activation of the effector protease, caspase-3. The Bcl-2 protein family regulates the intrinsic apoptosis pathway. (A) Under normal conditions, in which there is a proper balance between proapoptotic and antiapoptotic proteins, a cell death signal triggers changes in the mitochondrion (the primary organelle for initiating the intrinsic pathway), such as the formation of a Bax-Bax protein dimer in the outer mitochondrial membrane that facilitates release of cytochrome c. Once in the cytosol, cytochrome c binds and activates Apaf-1 protein, allowing it to bind and activate procaspase-9. Active caspase-9 has been shown to directly cleave and activate caspase-3, which leads to cell death. (B) Under conditions of Bcl-2 overexpression, the Bcl-2 protein, which resides in the mitochondrial outer membrane, blocks the apoptosis initiation process, such as by disrupting the Bax-Bax dimer. Release of cytochrome c in response to a cell death signal is prevented, and there is no initiation of the intrinsic apoptosis cascade. Reduction or removal of Bcl-2 protein by oblimersen allows restoration to A, resulting in apoptosis in response to a cell death signal. Adapted and reprinted by permission from ©American Society for Investigative Pathology, in Reed, J.C. (2000).Warner-LambertParke-Davis Award Lecture. Mechanisms of apoptosis. Am J. Pathol. 157, 1415–1430. Also adapted and reprinted by permission from *Nature* (www.nature.com), in Nicholson, D.W. (2000). From bench to clinic with apoptosis-based therapeutic agents. Nature 407, 810–816, Macmillan Publishers, Ltd.

mRNA interaction (Weiner et al., 1997; Wooldridge et al., 1997; Ballas et al., 1996; Krieg et al., 1995; Krieg, 2002). By using appropriate control oligonucleotides (e.g., sense, missense, reverse sequence, one-base and two-base mismatch missense) and various immunodeficient animal strains, researchers have attempted to separate therapeutic effects that can be attributed to the specific downregulation of target mRNA and protein from nonspecific effects.

AS-ONs DIRECTED AT Bcl-2

Although the exact molecular mechanism of action of Bcl-2 protein is not fully defined, AS-ONs can target a protein even when the biochemical mechanism of action is unknown and conventional small molecule inhibitors are difficult to obtain (Nicholson, 2000). AS-ONs against Bcl-2 in a human leukemia cell line were first used in 1990 (Reed et al., 1990b). Although this and subsequent studies demonstrated that Bcl-2 AS-ONs could promote *in vitro* cell death and reduce cell growth, their potency was limited because of poor uptake, intracellular compartmentalization, and limited accessibility of the AS-ON to its intended hybridization site on target *bcl-2* mRNA molecules (Reed, 1997; Tamm et al., 2001). Organisms have developed a variety of nuclease enzymes to destroy rogue DNA strands both inside and outside the cell. Development of therapeutic AS-ON molecules has required appropriate chemical modifications to confer nuclease resistance, along with a favorable pharmacokinetic profile (Agrawal et al., 1991; Raynaud et al., 1997). Modifications in the phosphodiester AS-ON backbone have yielded molecules now under clinical development. Phosphorothioates (sulfur substituted for one of the phosphodiester oxygens) (PS) are the most widely studied second-generation AS-ONs. PS-AS-ONs are nuclease resistant and capable of entering the cell and demonstrate good hybridization, pharmacokinetics, and minimal nonsequence-dependent effects or toxicities at concentrations required to downregulate the target mRNA. Additionally, PS-AS-ONs have demonstrated activity in free form *in vivo*, possibly due to interaction with blood lipoproteins (Jansen et al., 1998; Monia et al., 1996). Nonsequence-dependent PS-

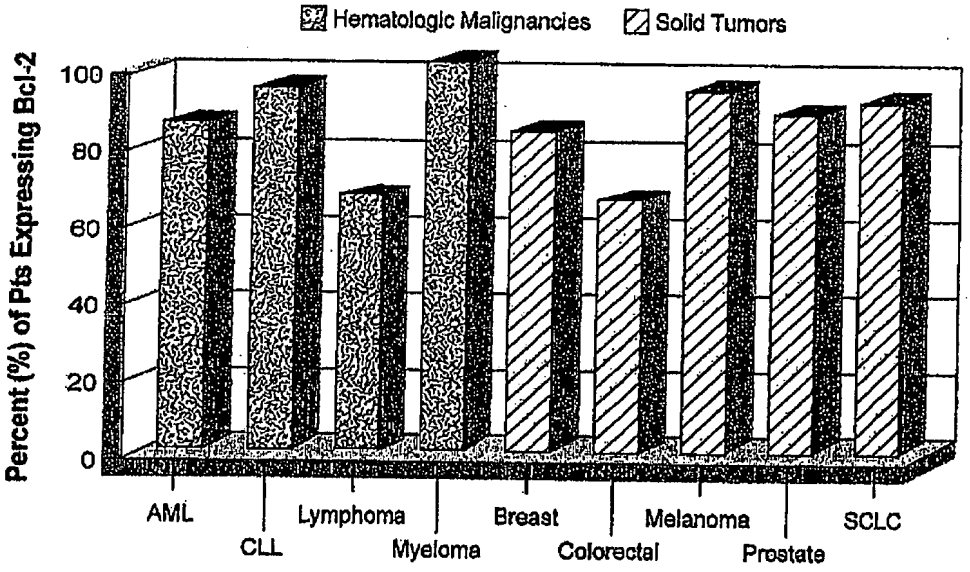

FIG. 2. Proportion of patients whose tumor cells at the time of diagnosis tested positive for Bcl-2 expression/overexpression, as reported in individual studies. Tissue samples were assessed using immunohistochemistry or flow cytometry. Data from Karakas et al. (1998), Lazaridou et al. (2000), Chen et al. (1997), Puthier et al. (1999), Lanzafame et al. (1998), H.-B. Yang et al. (1999), Jiang et al. (1995), Cerroni et al. (1995), and Sullivan et al. (1998). AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; SCLC, small-cell lung cancer.

AS-ON toxicities (e.g., hypotension, bradycardia, transient prolongation of activated partial thromboplastin time) have been observed at PS-AS-ON doses higher than those recommended for clinical trials (Kuss and Cotter, 1999). These toxicities have been reversible with discontinuation of drug infusion and are related to plasma drug levels. Oblimersen sodium (G3139, Genasense™) (Genta Inc., Berkeley Heights, NJ) is a PS-AS-ON that has been optimized for Bcl-2 downregulation and is in clinical development for a variety of oncology indications.

OBLIMERSEN: A Bcl-2 AS-ON

Chemistry

Oblimersen sodium (CAS No. 190977-41-4) is a fully phosphorothioated 18-mer AS-ON (5′-d(P-thio)TCT-CCC-AGC-GTG-CGC-CAT-3′) complementary to the first 6 codons of the ORF of the *bcl-2* mRNA sequence (Fig. 4) (Cotter, 2000; Nicholson, 2000). The formation of a heterologous PS-AS-ON-mRNA duplex targets the *bcl-2* mRNA for cleavage by RNase H, theoretically leaving the oblimersen molecule intact to bind to another *bcl-2* mRNA.

Oblimersen was identified as the most biologically active Bcl-2 antisense sequence from among a series of 40 PS-ONs 18- to 20-mers designed to recognize target sites distributed from 750 bases upstream of the initiation codon to the 3′-end of the published cDNA Bcl-2 sequence (Fig. 5) (Genta Incorporated, unpublished observations). Oligonucleotides were evaluated for downregulation of *bcl-2* mRNA expression in cultured T-24 human bladder carcinoma cells or MCF-7 human breast adenocarcinoma cells. Oblimersen (targeting positions 1–18 of the ORF) was the most active oligonucleotide, reducing *bcl-2* mRNA expression to near the lower limit of detection. Within the ORF, two other biologically active oligonucleotides (targeting positions 422 and 717) were identified. Additionally, there are two highly accessible regions in the *bcl-2* mRNA 3′-untranslated region (3′-UTR), from positions 1103 to 1218 and from positions 1846 to 2258, where all tested oligonucleotides demonstrated significant antisense activity.

To test for nonspecific oligonucleotide or immunologic effects, several control oligonucleotides have been used to compare responses to those of oblimersen in preclinical studies (Table 1). All are fully phosphorothioated 18-mer AS-ONs.

Pharmacokinetics

Preclinical experience. The pharmacokinetics of oblimersen have been studied in mice and humans (Table 2). Studies in mice demonstrate that oblimersen is rapidly distributed and slowly eliminated from plasma when administered via a single intravenous (i.v.) bolus, exhibiting a terminal half-life of 11 hours (Raynaud et al., 1997). Oblimersen pharmacokinetics follow a three-compartment model, and the agent is highly protein bound (98% at 5 minutes) (Raynaud et al., 1997), which is consistent with the high affinity that PS-ONs exhibit for albumin and α_2-macroglobulin (Bigelow et al., 1990; Zhang et al., 1995). Oblimersen does not cross the blood-brain barrier (Raynaud et al., 1997).

When oblimersen was administered via slow subcutaneous (s.c.) infusion in mice, plasma steady state was reached by day 3, which is consistent with rapid absorption and a terminal elimination half-life of 22 hours (Raynaud et al., 1997). Following s.c. infusion, about 50% of the parent compound was pro-

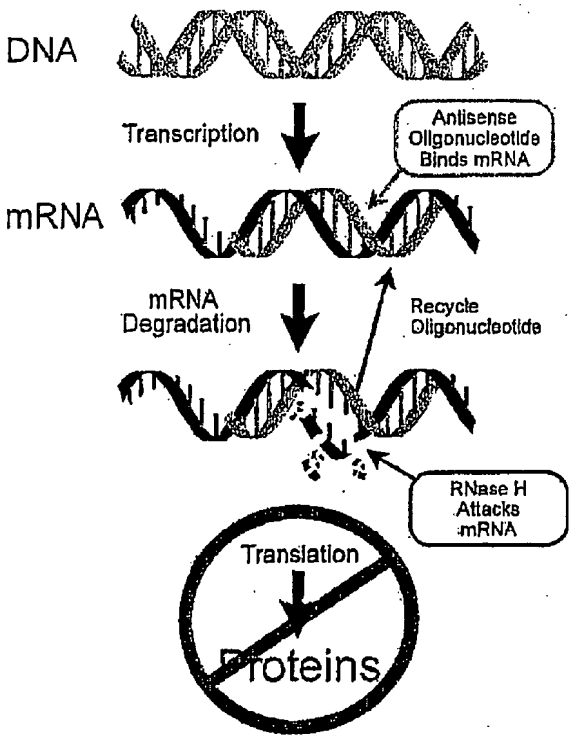

FIG. 3. Antisense mechanism of action decreases a target protein at an early stage. Therapeutic antisense oligonucleotides (AS-ONs) are designed to hybridize to and inactivate a specific mRNA that would produce a disease-relevant protein product. The presence of a double-stranded hybrid structure can prevent translation of the mRNA by blocking ribosome binding or progression. Furthermore, hybrids consisting of the mRNA strand with certain synthetic AS-ON are recognized by RNaseH, which degrades only the RNA portion, sparing the AS-ON. Thus, the AS-ON can function catalytically. Traditional small molecule drugs work at a later stage by inactivating a disease-relevant target protein after it has been produced.

tein bound at steady state, with significantly more parent drug reaching the tissues and bone marrow. The primary elimination route following both i.v. and s.c. administration in mice appears to be renal, with greater metabolism and elimination of the parent compound occurring with i.v. bolus administration.

When oblimersen was administered in combination with doxorubicin in SCID mice bearing a human breast cancer xenograft, increased Cmax, higher area under the plasma concentration-time curve (AUC), and a 9-fold lower plasma clearance were observed. The rate of oblimersen accumulation in organs was dependent upon steady-state levels in plasma and the presence of coadministered doxorubicin (Lopes de Menezes and Mayer, 2002).

Clinical experience. The pharmacokinetics of oblimersen have been evaluated recently in patients with lymphoma (Waters et al., 2000b), acute leukemia (Marcucci et al., 2001), malignant melanoma (Jansen et al., 2001), hormone-refractory prostate cancer (de Bono et al., 2001; Morris et al., 1999, 2002; Scher et al., 2000), colorectal cancer (Ochoa et al., 2001a,b), and assorted solid tumors (Chen et al., 2000) (Table 2). For each tumor type and regimen with doses greater than 2–3 mg/kg per day, steady-state plasma concentrations of ≥ 1 μg/mL were consistently achieved, a concentration at which oblimersen was found to be bioactive in animals (Raynaud et al., 1997).

Steady-state plasma concentrations of oblimersen were observed approximately 48 hours after initiation of a continuous s.c. infusion for 14 days by a portable infusion pump in 21 patients with advanced lymphoma (Waters et al., 2000b). Plasma levels correlated linearly with dose ($p = 0.002$), increasing from a mean of 0.45 μg/ml for the 36.8 mg/m^2 group to 5.63 μg/ml for the 195.8 mg/m^2 group. Plasma levels associated with dose-limiting toxicity in this population of lymphoma patients were >4 μg/ml, establishing the maximum tolerated dose (MTD) using this mode of delivery and schedule as 147 mg/m^2 per day (approximately 4.1 mg/kg per day). The AUC ranged from 107 to 1200 μg/ml/h. The mean plasma elimination half-life was 7.46 hours, with no difference in half-life observed between dose levels.

In 24 patients with malignant melanoma receiving a 5 or 14 day continuous i.v. infusion of 0.6–6.5 mg/kg oblimersen per day over 14 days or 5, 6, and 9 mg/kg per day over 5 days followed by a 1 hour infusion of dacarbazine (1000 mg/m^2), mean steady-state plasma oblimersen concentrations were generally reached and maintained after one day (Jansen et al., 2001). Mean concentrations ranged from 1 to 8 μg/ml, correlating linearly with delivered dose and unaffected by dacarbazine administration. At doses ≥ 1.7 mg/kg per day, steady-state plasma concentrations exceeded concentrations of 1 μg/ml. The drug was excreted primarily unchanged via the kidney.

Similar pharmacokinetic parameters have been observed in 16 patients with hormone-refractory prostate cancer who received 5–7 mg/kg oblimersen per day as a 5-day continuous i.v. infusion, followed by a 1-hour i.v. infusion of 60–100 mg/m^2 docetaxel, with the combination regimen repeated every 21 days (de Bono et al., 2001). No added toxicity was seen with the addition of oblimersen to docetaxel. Plasma oblimersen concentrations consistently exceeded 1 μg/ml, with steady-state concentrations averaging 3.09 and 5.36 μg/ml at the 5 mg/kg and 7 mg/kg per day dose levels, respectively (de Bono et al., 2001). These findings are consistent with those reported from a phase I/II study in which patients with solid tumors, the majority of whom (23 of 35) had prostate cancer, received ei-

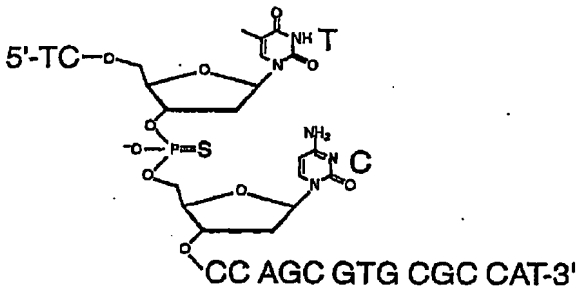

FIG. 4. Partial molecular structure of oblimersen sodium. The sequence of 18 nucleotides is presented in the 5'- to 3'-schematic convention, with a portion of the molecular structure expanded to show the nucleosides thymidine (T) at position 3 and cytodine (C) at position 4, joined by a phosphorothioate linkage.

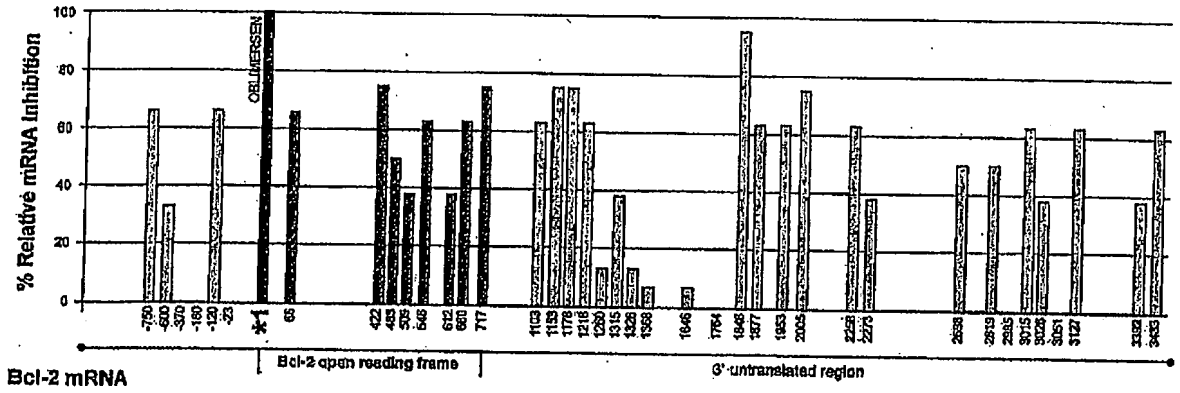

FIG. 5. Gene-walking analysis of *bcl-2* mRNA. T-24 or MCF-7 cells were plated and allowed to grow and recover to an initial density of 75%–85% before being transfected for 17 hours with various AS-ONs (200–500 nM, combined with cationic lipids optimized for each cell line). Reverse transcription-polymerase chain reactions (RT-PCR) were performed on total RNA isolated using primer sequences for Bcl-2 and β-actin (as normalization standard). Normalized RT-PCR data for 40 AS-ONs are shown as % inhibition of *bcl-2* mRNA expression relative to oblimersen (G3139) activity (100%) and plotted as the 5'-end binding position along the *bcl-2* mRNA of each AS-ON.

ther oblimersen alone at doses from 0.6 mg/kg per day for 14–21 days or 4.1, 5.3, or 6.9 mg/kg oblimersen per day followed by oblimersen plus i.v. paclitaxel 100 mg/m^2 weekly for 3 weeks (Morris et al., 2002; Scher et al., 2000). The observed plasma half-life was 2 hours and was dose independent. Steady-state plasma concentrations were 3–4, 4–5, and 7–8 μg/ml for oblimersen doses of 4.1, 5.3, and 6.9 mg/kg per day, respectively.

OBLIMERSEN: PRECLINICAL AND CLINICAL INVESTIGATIONS

The rationale for using Bcl-2 antisense in cancer derived from the central role of the Bcl-2 protein in apoptosis. Bcl-2 acts as a negative regulatory factor at the initiation stage of the apoptotic process following receipt of a death signal (i.e., dimerization of Bax protein) that has been triggered by some other stimulus (e.g., cellular damage, cytotoxic chemotherapy). A therapy-enhancing strategy depends on combining oblimersen with other anticancer agents to inflict more effective cellular damage. The clinical development program for oblimersen is based on the premise that downregulation of Bcl-2 can be employed to enhance the sensitivity of cancers to the apoptotic effects of other active anticancer agents. Preclinical observations concerning the role of *bcl-2* in tumor cell lines and animal xenograft models have demonstrated oblimersen is biologically active, both as a single agent and in combination with standard chemotherapeutic agents. Parameters included enhanced cellular apoptosis, tumor inhibition, tumor regression or elimination, and increased xenograft host survival (Table 3 and Fig. 6). Oblimersen has been studied in a variety of hematologic malignancies and solid tumors.

Hematologic malignancies

Non-Hodgkin's lymphoma. PRECLINICAL EXPERIENCE. Preclinical studies confirm that the Bcl-2 protein confers chemotherapy resistance in lymphoma (Schmitt et al., 2000). Bcl-2 AS-ONs specifically downregulated Bcl-2 protein and induced apoptosis in lymphoma cell lines (Kitada et al., 1993; Tormo et al., 1998; Obasaju and Smith, 2001). Several *in vitro* studies also showed that AS-ONs targeted against human *bcl-2* RNA reduced levels of Bcl-2 protein, such as in an NIH-3T3 fibroblast cell line infected with a recombinant retrovirus containing human *Bcl-2* cDNA, in a t(14;18)-containing lymphoma cell line SU-DHL-4, and in a transformed follicular lymphoma cell line having the t(14;18) translocation and inappropriately high expression of Bcl-2 protein (Kitada et al., 1993; Tormo et al., 1998).

A number of *in vivo* xenograft studies have been conducted to evaluate the activity of oblimersen as a single agent in lymphoma (Table 3 and Fig. 6). Ten million DoHH2 cells were inoculated i.v. into SCID mice that lacked functional B or T cells

TABLE 1. NUCLEOTIDE SEQUENCES OF OBLIMERSEN AND VARIOUS CONTROL OLIGONUCLEOTIDES USED IN PRECLINICAL RESEARCH

Oligonucleotide (properties)	*Nucleotide sequence*
Oblimersen (G3139, antisense to Bcl-2)	5'-TCT CCC AGC GTG CGC CAT-3'
G3622 (Reverse polarity sequence)	5'-TAC CGC GTG CGA CCC TCT-3'
G4126 (2-base mismatch sequence)	5'-TCT CCC AGC ATG TGC CAT-3'
G4232 (m^{5}C modification in CpG motifs)[a]	5'-TCT CCC AGm^{5}C GTG m^{5}CGC CAT-3'

[a]m^{5}C, 5-methyl deoxycytosine modification.

TABLE 2. PHARMACOKINETICS OF OBLIMERSEN

Administration route/ tumor type	Oblimersen regimen: Dose (mg/kg/day)	Oblimersen regimen: Duration (days)	AUC[a] (μg·h/ml)	Mean plasma C_{ss} (μg/ml)	Mean plasma $T_{1/2}$ (h)	MRT (h)	V_{ss} (ml/g)	Cl (ml/h)
Mouse[b]								
i.v.	5	Single dose	10	NR	11	4.41	33	7.7
s.c.	5	7	160	1	22	36	4.7	NR
SCID mouse[c]								
i.p.	5	3	11.2	NR	209.7	NR	7.7	0.4
Human								
s.c. (CI; NHL)[d]	[4.6–195.8][e]	14	107–1,200[f]	0.45–5.63[f]	7.46	NR	NR	1.71–9.62
i.v. (CI; melanoma)[g]	0.6–6.5 or 5–9	14 or 5	NR	1–8[f]	NR	NR	NR	NR
i.v. (CI; prostate cancer)[h]	5–7	5	NR	3.09–5.36[f]	NR	NR	NR	NR
i.v. (CI; prostate cancer)[i]	0.6–6.9 or 4.1–6.9 + P	14–21	894–2550	O:<1–7.6 O+P: 3–8	2			
i.v. (CI; breast cancer)[j]	1–4	21	NR	>1.5[k]	NR	NR	NR	NR
i.v. (CI; colorectal cancer)[l]	3–7	7	NR	>5[m]	NR	NR	NR	NR

[a]AUC, area under the plasma concentration-time curve; C_{ss}, steady-state plasma concentration; $T_{1/2}$, terminal elimination half-life; MRT, mean residence time; V_{ss}, volume of distribution; Cl, clearance; i.v., intravenous; NR, not reported; s.c., subcutaneous; CI, continuous infusion; NHL, non-Hodgkin's lymphoma; O, oblimersen; P, paclitaxel.

[b]Raynaud et al., 1997.

[c]Lopes de Menezes and Mayer, 2002. Oblimersen given in combination with doxorubicin i.v. 5 mg/kg (administered 1 hour before oblimersen).

[d]Waters et al., 2000b.

[e]Oblimersen doses given as mg/m² per day.

[f]Increased linearly with dose. With doses ≥1.7 mg/kg/day, C_{ss} exceeded biologically relevant concentrations of 1 μg/ml.

[g]Jansen et al., 2001. Oblimersen was administered alone or in combination with dacarbazine 80 mg/kg per day i.p. × 5 days (days 12–16).

[h]de Bono et al., 2001. Oblimersen was administered alone or in combination with docetaxel 60–100 mg/m² i.v. over 1 hour on day 5.

[i]Morris et al., 1999, 2002; Scher et al., 2000. Oblimersen doses ≥4.1 mg/kg per day were administered alone or in combination with paclitaxel 100 mg/m² on days 8, 15, and 22 during the second and third treatment cycles.

[j]Chen et al., 2000.

[k]At a dose level of 3 mg/kg per day, in combination with docetaxel.

[l]Ochoa et al., 2001a,b.

[m]When administered at 5 and 7 mg/kg per day in combination with irinotecan.

but retained natural killer (NK) cell activity and nonobese diabetic SCID (NOD/SCID) mice that lacked NK, B, and T cell activity (Waters et al., 2000a). These mice were treated with oblimersen or control oligonucleotides by s.c. infusion for 14 days, commencing on day 8 after tumor inoculation. All mice in each group that were treated with control oligodeoxynucleotides developed overt NHL. In comparison, only 3 of 18 SCID mice and 1 of 6 NOD/SCID mice that received a 14-day s.c. infusion of oblimersen developed NHL, even when analyzed by PCR for the characteristic t(14;18) break point (Waters et al., 2000a; Cotter et al., 1999).

In another study, escalating doses of oblimersen, cyclophosphamide, and the combination were evaluated in SCID mice bearing a systemic human DoHH2 lymphoma xenograft (Klasa et al., 2000). Results confirmed that oblimersen downregulated Bcl-2 expression *in vitro* and that treatment with oblimersen alone resulted in prolonged median survival and cure of some animals (Fig. 7). This effect was dose and schedule dependent, with no long-term survivors after an oblimersen dose of 5 mg/kg daily for 14 consecutive days. However, long-term survival was >40% when the oblimersen dose was increased to 12.5 mg/kg on the same daily schedule or either 5 or 12.5 mg/kg was administered for 14 treatments on alternate days (28-day schedule). Similarly, cyclophosphamide treatment alone resulted in no long-term survivors at lower doses but cures at high doses. The addition of oblimersen to low-dose cyclophosphamide (35 mg/kg) resulted in the cure of all animals (Table 3 and Figs. 6 and 7). The interaction between the two agents demonstrates dose-response correlations. For the two low doses of cyclophosphamide tested, increasing the oblimersen dose from 2.5 to 5 mg/kg resulted in longer median survivals and an increase in number of long-term survivors. When an ineffective cyclophosphamide dose (15 mg/kg, median survival 36 days; no long-term survivors) was combined with a modestly effective oblimersen dose (2.5 mg/kg, 61-day median survival; 16% long-term survivors), the median survival increased to 72 days, and 50% of animals survived long term. Mice sacrificed at 90 days showed no histologic evidence of disease using immunoperoxidase staining for Bcl-2 or molecular detection of *bcl-2* by PCR. These findings suggest that modest chemotherapy doses could be effective without increasing toxicity when combined with an AS-ON. Treatment of mice

TABLE 3. EVALUATION OF OBLIMERSEN AGAINST HUMAN TUMOR XENOGRAFTS IN MICE

Malignancy	*Cell line*	*Dosage*	*Comments*
Lymphoma	DoHH2	Oblimersen 14-day s.c. infusion[a]	3/18 SCID mice and 1/6 NOD/SCID mice treated with oblimersen had detectable NHL, using PCR for t(14;18) break point compared with all mice in both groups receiving control oligonucleotides.[b]
Lymphoma	DoHH2	Oblimersen 5 mg/kg i.p. × 14 doses given over 18 or 28 days ± cyclophosphamide 35 mg/kg i.p. on days 4, 8, 12	Animals receiving oblimersen showed a significant ($p < 0.000001$) increase in median survival from 33 days to 62 days compared with control. Oblimersen plus cyclophosphamide led to eradication of DoHH2 cells *in vivo* and cure of animals.[c]
EBV-associated lymphoproliferative disorder	LCL, Sweig cells	Oblimersen 10 mg/kg/day × 12 days i.p.	5/7 oblimersen-treated LCL animals remained alive and without clinical signs of tumor for 185 days. In comparison, all animals in control groups died of gross tumor by day 46. Survival of oblimersen-treated Sweig cell animals was significantly prolonged compared with the control group ($p < 0.001$).[d]
EBV-associated lymphoproliferative disorder	LCL, Sweig cells	Oblimersen 10 mg/kg/d × 12 days i.p. ± rituximab 10 mg/kg i.p. weekly × 4	Treatments delayed until day 15 to establish tumors. 5/7 SCID mice receiving combination remained tumor-free until they were sacrificed at day 191. In contrast, 7/7 rituximab-treated mice and 6/7 oblimersen-treated mice died with tumor by day 80.[d]
Leukemia	TF-1-STI571[R]	Oblimersen 7 mg/kg/day i.p. × 14 days	Nearly all oblimersen-treated mice survived >6 months and had reduced tumor volume, with 3/5 demonstrating a complete regression, whereas untreated mice died within 10 weeks. Cells harvested from oblimersen-treated animals demonstrated sensitivity to subsequent treatment with imatinib mesylate, daunorubicin, cytosine arabinoside, and etoposide, with additive or synergistic effect in the induction of apoptosis.[e]
Melanoma	581A2	Oblimersen 5 mg/kg/day s.c. × 14 days	Mean tumor weight for oblimersen-treated and control-treated mice was 0.39 ± 0.12 g and 0.96 ± 0.20 g, respectively (59% decrease in tumor weight for oblimersen vs. control). Oblimersen enhanced the number of apoptotic cells within sections of melanoma cells from 0.69% (saline) to 3.38% (oblimersen).[f]
Melanoma	581A2	Oblimersen 5 mg/kg/day s.c. × 14 days then dacarbazine 80 mg/kg/day i.p. × 5 days	Mean tumor weight for oblimersen/dacarbazine-treated and control-treated mice was 0.02 ± 0.02 g and 1.53 ± 0.15 g, respectively (99% decrease in tumor weight for oblimersen vs. control). Mean tumor weight for dacarbazine-treated mice was 0.44 ± 0.10 g. Complete tumor ablation occurred in 3/6 and 7/7 animals in repeat experiments.[f]
Prostate cancer	PC3	Oblimersen 10 mg/kg s.c. × 7 days plus docetaxel 6 mg/kg i.v. days 5,7,9	Oblimersen alone led to tumor growth delay and increased survival. Oblimersen plus docetaxel led to marked tumor reduction, with 3/10 mice having no evidence of tumor regrowth and 4/10 surviving >60 days. No long-term survivors observed with either agent alone.[g]

Breast cancer	MDA-MB-231, MDA-MB-435, MDA-MB-361	Oblimersen ± subcytotoxic doses of docetaxel, paclitaxel, or cisplatin[h]	Oblimersen treatment alone inhibited tumor formation by 60%–90% *in vivo* in all xenografts. Synergistic and complete tumor regression was observed when oblimersen was combined with docetaxel, paclitaxel, or cisplatin, with mice remaining tumor free for >5 months. No effects were observed with combined reverse or mismatch control oligonucleotide treatment.[h]
Breast cancer	MDA435/LCC6	Oblimersen 5–10 mg/kg i.p. days 3–7, 10–14, 17–21 ± free or liposomal doxorubicin 5–10 mg/kg i.v. weekly × 1, 3, or 6 weeks. Treatment initiated on day 3 or day 17 after cell inoculation	Day 35 tumor weights were 0.44 g and 0.17 g in mice receiving early treatment with control and oblimersen, respectively. Treatment of established tumors exerted minimal and transient antitumor effects despite prolonged treatment. Combination of oblimersen with either free (early treatment models) or liposomal doxorubicin (larger solid tumors) showed maximum growth suppression compared with individual treatments alone.[i]
Colon cancer	GEO	Oblimersen 10 mg/kg/dose i.p. 5 days per week × 3 weeks (alone or in combination with AS RIα)	By day 35, oblimersen caused 80%–90% inhibition of tumor growth compared with control untreated mice. Growth resumed after the end of treatment similar to growth rate in untreated mice. A significant ($p < 0.001$) increase in survival was observed with oblimersen vs. control. Combination treatment with oblimersen plus AS RIα resulted in prolonged inhibition of tumor growth and increased survival compared with either agent alone.[j]
NSCLC	NCI-H460	Oblimersen 3, 5, 8 mg/kg s.c. b.i.d. day 8 to day 14 post tumor implantation or Oblimersen 5 mg/kg s.c. b.i.d. day 8 to day 14 post tumor implantation plus docetaxel 7.5–24.7 mg/kg i.v. days 9,12,15	High antitumor activity observed with oblimersen 5 mg/kg per dose plus docetaxel 20.8 mg/kg per injection, with a 3.1 log cell kill and complete regression in 4 of 7 mice, compared with lower cell kill and no regression using either agent alone.[k]
Gastric cancer	Kato III, N87	Oblimersen 10 mg/kg/day × 28d ± cisplatin i.p. 9 mg/kg day 7	Combination treatment led to >70% reduction in mean tumor volume ($p < 0.006$) and in increase in survival vs. either single agent or control.[l]
Merkel cell carcinoma	MC MA11	Oblimersen 10 mg/kg/day s.c. continuous infusion × 28 days	Significant or complete tumor ablation in mice receiving oblimersen compared with saline, reverse control, or mismatch oligonucleotides ($p < 0.05$), along with a reduction in Bcl-2 levels and a significant ($p < 0.05$) increase in the mean apoptosis.[m]

[a]s.c., subcutaneously; SCID, severe combined immunodeficiency; NOD, nonobese diabetic; NHL, non-Hodgkin's lymphoma; PCR, polymerase chain reaction; i.p., intraperitoneal; EBV, Epstein-Barr virus; AS RIα, antisense RIα; NSCLC, non-small cell lung cancer; b.i.d., twice daily.
[b]Waters et al., 2000a.
[c]Klasa et al., 2000, 2001.
[d]Guinness et al., 2000; Lacy and Loomis, 2002.
[e]Tauchi et al., 2002.
[f]Jansen et al., 1998.
[g]Tolcher et al., 2001.
[h]Administration route not specified; D. Yang et al., 1999.
[i]Lopes de Menezes et al., 2000.
[j]Tortora et al., 2001.
[k]Vrignaud et al., 2002.
[l]Wacheck et al., 2001a,b.
[m]Schlagbauer-Wadl et al., 2000.

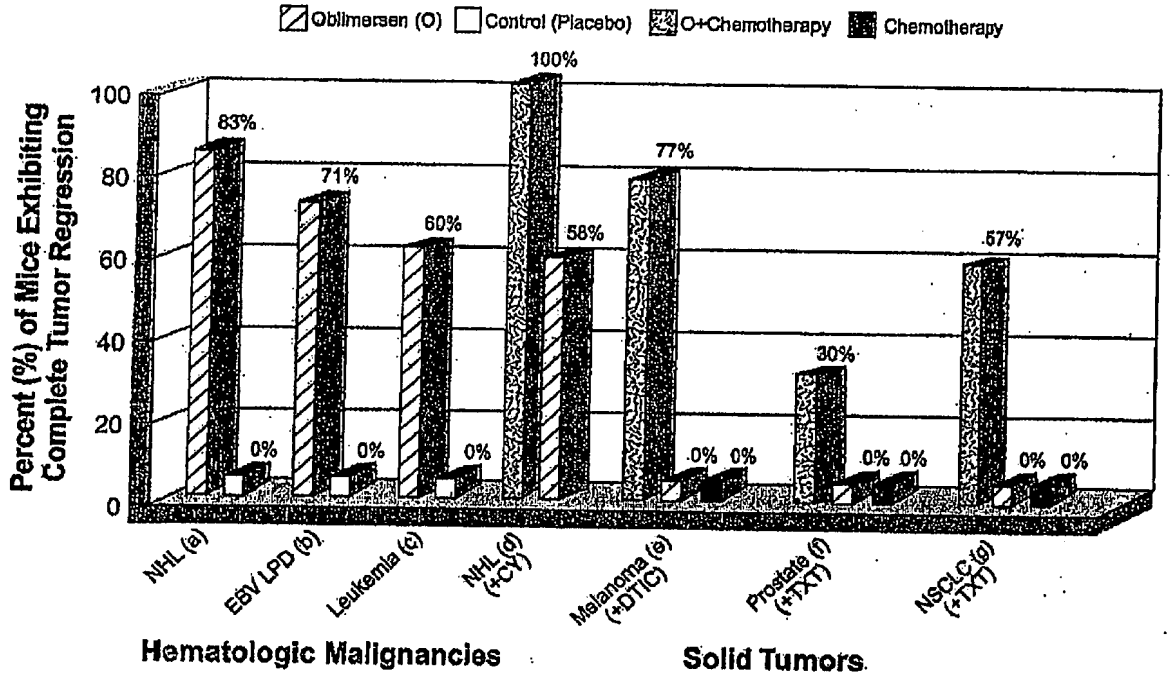

FIG. 6. Percent of mice exhibiting complete xenograft tumor regression following treatment with oblimersen plus chemotherapy compared with single agents, or oblimersen alone compared with control. EBV LPD, Epstein-Barr virus-associated lymphoproliferative disorders; NHL, non-Hodgkin's lymphoma; CY, cyclophosphamide; DTIC; dacarbazine; TXT, docetaxel; NSCLC, non-small cell lung cancer. Data from (a) Waters et al., 2000a, (b) Guinness et al., 2000, (c) Tsuchi et al., 2002, (d) Klasa et al., 2000, 2001, (e) Jansen et al., 1998, (f) Tolcher et al., 2001, and (g) Vrignaud et al., 2002.

deficient in NK cell activity (perforin deficient) provided similar results, supporting the view that stimulation of the host immune system by oblimersen does not significantly affect elimination of lymphoma (Klasa et al., 2000). Other effector cells may be intact in this model. Oligonucleotides with CpG domains may act on plasmacytoid precursor dendritic cells (ppDC) to trigger release of interferon-α (IFN-α) and trigger TNF, interleukin-6 (IL-6), and IL-12 release from peripheral blood mononuclear cells (PBMC) and purified monocytes (Koziolkiewicz et al., 2001).

In vitro treatment of immortalized lymphoblastoid B (LCL) cells, expressed in the majority of Epstein-Barr virus (EBV)-associated posttransplant lymphoproliferative disorders, with single-agent oblimersen was associated with decreased expression of Bcl-2 protein, inhibition of proliferation, and stimulation of apoptotic cell death (Guinness et al., 2000). In addition, treatment of LCL-bearing SCID mice with oblimersen completely prevented or significantly delayed development of fatal EBV-positive lymphoproliferative disease (Table 3 and Fig. 6). In another SCID mouse study, a delayed treatment schedule was used in order to detect enhanced antitumor effect of combined oblimersen and rituximab (Rituxan, Genentech, Inc., South San Francisco, CA) against established LCL tumors (Lacy and Loomis, 2002). SCID mice ($n = 7$ per group) were injected i.p. with 20 million LCLs (Sweig) on day 0. On day 15, treatment was initiated with oblimersen alone (10 mg/kg/day over 12 days in five divided doses i.p. at 72-hour intervals), rituximab alone (10 mg/kg i.p. weekly × 4), or oblimersen plus rituximab (same dose and schedule used for monotherapy). Untreated control animals died with tumor 43–47 days after LCL injection. The median survival of the two monotherapy arms was prolonged compared with the untreated control arm, but all animals in both monotherapy arms died with tumor. In contrast, when oblimersen was combined with rituximab, 5 of 7 animals remained tumor free at the time of sacrifice (150 days), and median survival of the combined treatment arm was 150+ days (Table 3).

CLINICAL EXPERIENCE. High Bcl-2 expression has been demonstrated in up to 55% of patients with large-cell NHL, and three studies evaluating the prognostic significance of Bcl-2 expression in these patients confirmed its importance as an independent prognostic marker for shorter disease-free survival and higher relapse rates (Hermine et al., 1996; Hill et al., 1996; Gascoyne et al., 1997). A phase I trial was conducted to evaluate the safety and activity of oblimersen as a single agent in patients with NHL. Twenty-one patients received a 14-day s.c. infusion of oblimersen (doses ranging from 4.6 to 195.8 mg/m^2 per day) (Webb et al., 1997; Waters et al., 2000b). The MTD in this trial was 147.2 mg/m^2 per day (4.1 mg/kg per day). Dose-limiting toxicities included thrombocytopenia, hypotension, fever, and asthenia. There was 1 complete response, 2 minor responses, and 9 patients who experienced stable disease. The patient who achieved a complete response received a single 14-day course at 2 mg/kg per day and has maintained this response for longer than 5 years despite failing four prior therapies for follicular lymphoma. Bcl-2 protein was reduced in 7 of 16 as-

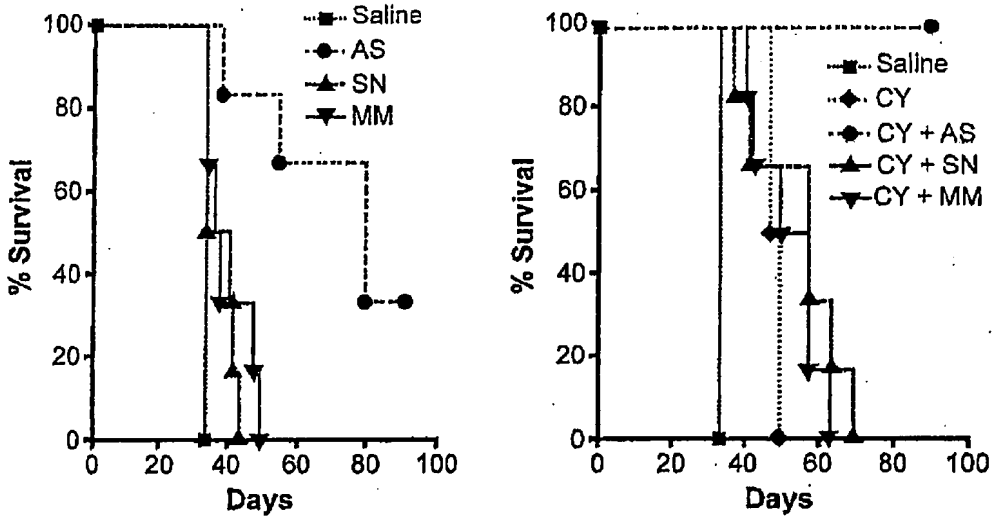

FIG. 7. Survival of cohorts of 6 mice receiving AS-ONs alone (5 mg/kg) (left) or AS-ONs combined with cyclophosphamide (35 mg/kg) (CY) (right). All 6 surviving animals receiving oblimersen with CY were sacrificed at 90 days, with no molecular evidence of disease detected. Saline, control animals; AS, oblimersen; SN, G3622 reverse sequence control; MM, G4126 2-base mismatch control. Reprinted by permission from Taylor & Francis, Ltd., in Klasa, R.J., List, A.F., and Cheson, B.D. (2001). Rational approaches to design of therapeutics targeting molecular markers. *In Hematology 2001. American Society Hematology Education Program Book.* (American Society of Hematology, Washington, D.C.), pages 443–462.

sessable patients. This trial demonstrated that oblimersen therapy is feasible in patients with NHL, with promising antitumor activity. These results suggest that oblimersen should be tested in combination with chemotherapy and other approaches, such as monoclonal antibody therapy.

Myeloid leukemias. PRECLINICAL EXPERIENCE IN CML. Oblimersen has been evaluated in a xenograft model for Philadelphia chromosome (Ph)-positive leukemia (Tauchi et al., 2002). Imatinib mesylate (STI-571, Gleevec®, Novartis AG, Basel, Switzerland) is an inhibitor of the Abelson kinase constitutively activated in the *bcr-abl* fusion gene product. In this study, nude mice were transplanted with imatinib mesylate-resistant *bcr-abl*-transformed TF-1 cells and then treated with placebo ($n = 5$) or oblimersen 7 mg/kg per day i.p. for 14 days ($n = 5$). All the untreated mice died within 10 weeks, whereas the majority of mice treated with oblimersen survived longer than 6 months and demonstrated reduced tumor volume (Table 3 and Fig. 6), with 3 of 5 oblimersen-treated mice demonstrating complete tumor regression. In addition, cells harvested from mice treated with oblimersen (7 mg/kg per day for 7 days) were more sensitive to further treatment with imatinib mesylate, daunorubicin, cytarabine, or etoposide, with each combination showing additive or synergistic activity in the induction of apoptosis. These findings support the ongoing evaluation of oblimersen, both alone and in combination with other chemotherapeutic agents in the treatment of myeloid leukemias.

CLINICAL EXPERIENCE IN RELAPSED AML. A phase I trial demonstrated that oblimersen 7 mg/kg per day for 10 days combined with full-dose fludarabine, cytarabine, and filgrastim (FLAG) was well tolerated (Marcucci et al., 2001). Nine of 18 (50%) evaluable patients demonstrated a response. Seven patients (39%) achieved a complete remission, and 2 additional patients cleared blasts from the marrow and blood but failed to recover normal neutrophil or platelet counts. It should be noted that several of the responders received less than full-dose FLAG therapy and only 4 mg/kg per day of oblimersen. Four of the 9 responders went on to receive a second consolidation course. Among the 9 responders, 4 were older than 60 years of age, 5 had previously received high-dose cytarabine therapy, and 1 had relapsed following an autologous stem cell transplantation. In the cohort that received oblimersen 7 mg/kg per day with full-dose FLAG, 3 of 6 patients achieved a complete remission. One patient remains in complete remission beyond 15 months following a single course of therapy. Duration of response ranged from 30 to 485+ days, with a median time to relapse of 122 days. Toxicities included central nervous system (CNS) bleeding ($n = 1$), fever, nausea, vomiting, hypocalcemia, hypophosphatemia, and fluid retention but were not dose limiting or clearly attributable to oblimersen. The MTD was not reached. The median times to neutrophil and platelet recovery were 23 days (range 8–38 days) and 39 days (range 21–56 days), respectively. These findings support the safety of adding oblimersen to combination chemotherapy for the treatment of patients with acute leukemia.

These results prompted initiation of an ongoing clinical trial of oblimersen plus gemtuzumab ozogamicin in elderly patients with relapsed AML. A pilot study of oblimersen combined with daunorubicin and cytarabine in patients over 60 years of age with newly diagnosed AML is underway.

Chronic lymphocytic leukemia. PRECLINICAL EXPERIENCE. High levels of Bcl-2 have been consistently demonstrated in B cell CLL lines and in cells taken from patients with CLL (Pepper et al., 1996; Hanada et al., 1993; Schena et al., 1992), suggesting a role for Bcl-2 in the pathogenesis and progression of this disease. In early preclinical studies, antisense oligonucleotides to *bcl-2* mRNA were shown to decrease Bcl-2 protein expression in a human pre-B cell leukemia cell line (Reed et al., 1990b) and to specifically inhibit *bcl-2* mRNA expression in B-

CLL cells (Pepper et al., 1999). Treatment with Bcl-2 antisense oligonucleotides targeting the same mRNA region as oblimersen also decreased Bcl-2 levels in CLL cell lines and in cells taken from patients (Joshi et al., 2001). In this study, Bcl-2 antisense oligonucleotides also significantly increased killing of CLL cells when combined with fludarabine and enhanced the immunologic attack from lymphokine-activated killer (LAK) cells. Recently, CLL cells obtained by CD19 selection of peripheral blood samples from patients were placed in culture for 72 hours with oblimersen or under control conditions. Bcl-2 expression and apoptosis were measured 24 hours later. Bcl-2 protein was markedly reduced by oblimersen treatment in a sequence-specific manner. Oblimersen 2 μM was more active than either fludarabine 50 μM or dexamethasone 1 μM as an inducer of apoptosis in this system, and pretreatment with oblimersen sensitized CLL cells to rituximab. A similar experiment in a follicular lymphoma cell line demonstrated even greater synergy between oblimersen and rituximab (Auer et al., 2001).

CLINICAL EXPERIENCE. Oblimersen is undergoing evaluation in a nonrandomized clinical trial as monotherapy for patients with relapsed or refractory CLL (O'Brien et al., 2001). Patients receive oblimersen as a continuous i.v. infusion for 5–7 days every 3 weeks. Patients treated with oblimersen at 5 or 7 mg/kg per day experienced high fever, hypotension, and hypoglycemia, as well as back pain requiring narcotics. This clearly demonstrates that patients with CLL are more sensitive to the side effects of oblimersen (O'Brien et al., 2001) compared with patients with solid tumors in whom these doses are generally well tolerated. A daily dose of 3 mg/kg appears to be well tolerated either as a single agent or combined with fludarabine and cyclophosphamide. The basis for the difference in MTD between CLL and NHL compared with solid tumors, acute myeloid leukemia, and myeloma is likely disease specific. Either tumor lysis or direct oligonucleotide immunostimulation of the malignant B cells may explain this distinct toxicity pattern (Decker et al., 2000, 2002). Oblimersen is also being evaluated in a multicenter, international, randomized clinical trial as combination therapy with fludarabine plus cyclophosphamide for second-line treatment of patients with refractory/relapsed CLL.

Multiple myeloma. PRECLINICAL EXPERIENCE. Studies using Bcl-2 antisense plasmids demonstrated that Bcl-2 plays a primary role in the development of resistance to dexamethasone-induced and paclitaxel-induced apoptosis in MM cells (Hu and Gazitt, 1996; Gazitt et al., 1998). To evaluate the potential applications of oblimersen in MM, human myeloma cell lines were incubated with oblimersen, resulting in a time-dependent and dose-dependent uptake of oblimersen into the cytoplasm and nucleus (van de Donk et al., 2000). These cells exhibited a time-dependent and dose-dependent, sequence-specific decrease in *bcl-2* mRNA by 48 hours, as well as >75% reduction in Bcl-2 protein levels in myeloma cells after 4 days of exposure, without significant change in actin or Bax proteins (van de Donk et al., 2000). In U266 myeloma cells (a high Bcl-2-expressing MM cell line), oblimersen induced decreases in Bcl-2 protein, enhancing apoptosis and cytotoxicity from doxorubicin (van de Donk et al., 2000).

In another study, the effects of oblimersen over 1–4 days were evaluated using 6 MM cell lines and 2 Bcl-2-transfected MM cell lines with varying degrees of baseline Bcl-2 expression (Gazitt et al., 2001). High Bcl-2-expressing MM cell lines demonstrated up to a 60% decrease in Bcl-2 protein levels after 4 days of incubation with oblimersen 5 μg/ml, which was associated with low levels of apoptosis. In contrast, in MM cell lines with relatively low initial Bcl-2 protein levels, extensive apoptosis was induced at a log lower concentration of oblimersen, along with essentially complete depletion of Bcl-2 protein. When all cell lines were pretreated with oblimersen 10 μg/ml for 3 days followed by cytotoxic chemotherapy (dexamethasone, paclitaxel, or adenovirus p53) for 2 additional days, the fraction of cells that underwent apoptosis significantly increased compared with the cytotoxic agent alone. For example, the fraction of high *bcl-2*-expressing cell lines (ARH-77, U266) undergoing apoptosis increased from 15%–20% with dexamethasone alone to 40%–80% with the combination. Similar results were reported with paclitaxel (from 10%–24% to 56%–85%) and adenovirus p53 (from 6%–12% to 40%–50%). Cell lines that had been rendered resistant to chemotherapy by *bcl-2* transfection demonstrated low levels of apoptosis and relatively small reductions in Bcl-2 proteins when treated with either chemotherapy or oblimersen alone but showed a marked increase in apoptosis and cytotoxicity when treated with the combination. Thus, additive and potentially synergistic effects were observed between oblimersen and chemotherapy in myeloma cells expressing both low and high levels of Bcl-2 and support clinical trials in cancer patients irrespective of the level of Bcl-2 expressed in their tumors.

CLINICAL EXPERIENCE. A randomized, open-label, multicenter trial has been initiated to compare time to disease progression, objective response rate, duration of response, safety, and survival for patients with relapsed or refractory MM receiving dexamethasone with or without oblimersen.

Solid tumors

Malignant melanoma. PRECLINICAL EXPERIENCE. Oblimersen and other Bcl-2 AS-ONs have been evaluated *in vitro* and in mice with human melanoma xenografts. Exposure of 518A2 melanoma cells *in vitro* to oblimersen at a concentration of 200 nM (in the presence of uptake-enhancing cationic lipids) resulted in an almost complete loss of *bcl-2* mRNA within 24 hours (Jansen et al., 1998). At concentrations of 25 nM, 50 nM, 100 nM, and 200 nM, oblimersen decreased Bcl-2 protein levels by 35%, 33%, 47%, and 61%, respectively, after 48 hours. Exposure to G3622 reverse sequence and G4126 2-base mismatch control oligonucleotides resulted in only modest inhibition of Bcl-2 protein expression (17%). Similar findings also were reported for two other human melanoma cell lines expressing Bcl-2 at high levels (Jansen et al., 1998).

These *in vitro* results prompted further evaluation of oblimersen in a SCID mouse s.c. xenotransplantation model (Jansen et al., 1998) (Table 3). In SCID mice with established 518A2 human melanomas, treatment with oblimersen 5 mg/kg per day s.c. for 14 days resulted in a significantly lower mean tumor weight (0.39 $\pm$ 0.12 g) compared with treatment using saline (0.96 $\pm$ 0.20 g), reverse sequence oligonucleotide (1.02 $\pm$ 0.37 g), or mismatch oligonucleotide (0.90 $\pm$ 0.18 g) ($p < 0.004$). Further, Western blot analysis demonstrated a 66%–72% decrease in Bcl-2 protein in the oblimersen group compared with a <19% reduction in the reverse sequence and mismatch oligonucleotide groups. Oblimersen increased the proportion of apoptotic cells within sections of 518A2 hu-

man melanoma xenografts from 0.69% (saline) to 3.38% (oblimersen group). When oblimersen was combined with dacarbazine in this SCID mouse model, complete ablation of the tumor occurred in 10 of 13 animals, compared with a reduction in mean tumor size but not eradication in 6 mice treated with dacarbazine alone (Figs. 6 and 8). These data established that oblimersen reduces Bcl-2 expression and enhances the efficacy of cytotoxic chemotherapy in human melanoma xenografts and support the evaluation of oblimersen in patients with malignant melanoma.

More recently, Wacheck et al. (2002) investigated whether immunostimulation could contribute to oblimersen's antitumor activity in this SCID mouse melanoma xenograft model. Oligonucleotides with CpG motifs and certain flanking regions can be immunostimulatory, and oblimersen contains two CpG sequences. It is widely accepted that 5-methylation of cytosine (m^5C) in the CpG motif abrogates immunostimulation (Krieg, 2002). The antitumor potential of oblimersen was compared with that of oligonucleotide G4232, which is identical to oblimersen except for substitution of m^5C for cytosine in th two CpG positions (Wacheck et al., 2002). Following treatment with saline, oblimersen, or G4232 at a dose of 5 mg/kg per day for 14 days by implanted miniosmotic pump, animals were sacrificed for tumor and plasma analysis. Oblimersen and G4232 similarly reduced tumor growth by about one third relative to the saline-treated group ($p < 0.05$), which resulted in similar increases in tumor cell apoptosis compared with saline-treated controls. There was evidence for an oblimersen immunostimulatory effect in this model. Mice treated with oblimersen had about a 3-fold increase in spleen weight and about a 6-fold increase in serum IL-12 values compared with mice treated with saline, both differences being highly statistically significant ($p < 0.001$). In contrast, mice treated with G4232 had mildly elevated spleen weight and serum IL-12 values that did not differ significantly from those of mice treated with saline. Thus, the antitumor effect of oblimersen appears to be predominantly a Bcl-2 antisense effect that is independent of immunostimulation, as oblimersen and its immune-silent counterpart G4232 produced similar tumor suppression and apoptosis induction.

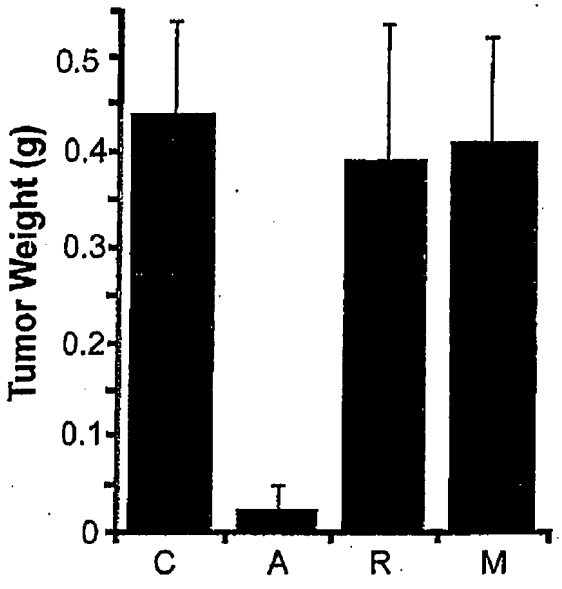

FIG. 8. Mean tumor weight (±SD) of melanoma tumors in SCID mice ($n = 6$ per group), following a 14-day infusion of saline (C), oblimersen (A), G 3622 reverse control (R), or G 4126 two-base mismatch oligonucleotides (M) plus dacarbazine on days 12–16. Mice were evaluated 21 days after initial cell inoculation. Reprinted by permission from *Nature Med* (www.nature.com) in Jansen, B., Schlagbauer-Wadl, H., Brown, B.D., Bryan, R.N., Van Elsas, A., Muller, M., Wolff, K., Eichler, H.G., and Pehamberger, H. (1998). Bcl-2 antisense therapy chemosensitizes human melanoma in SCID mice. Nature Med 4, 232–234.

CLINICAL EXPERIENCE. Clinically, aberrant Bcl-2 expression occurs in virtually all human melanoma (Selzer et al., 1998) and has been linked to chemotherapy resistance and shorter survival in patients (Grover and Wilson, 1996; Reed, 1999). A nonrandomized clinical study evaluating oblimersen plus decarbazine was performed in 24 patients with advanced malignant melanoma, including patients with disease resistant to decarbazine and other first-line regimens (Jansen et al., 2000, 2001). Oblimersen was administered in one of three schedules. One cohort received a continuous i.v. infusion for 14 days (daily doses ranging from 0.6 mg/kg to 6.5 mg/kg), with dacarbazine 200 mg/m^2 per day given for 5 days beginning on day 5. A second cohort received oblimersen 5.3–7.7 mg/kg per day given as twice daily s.c. injections combined with dacarbazine 800 mg/m^2 on day 5. The third cohort received daily oblimersen doses of 5, 7, 9, or 12 mg/kg by continuous i.v. infusion with dacarbazine 1000 mg/m^2 administered at the conclusion of oblimersen infusion on day 6. The treatment cycle was repeated every 28 days.

All combinations were well tolerated in this patient population. Hematologic toxicities were mild or moderate in severity and were similar to those observed with single-agent decarbazine therapy. Thrombocytopenia was dose limiting at an oblimersen dose of 12 mg/kg per day when combined with dacarbazine 1000 mg/m^2. Lymphopenia was the most frequently reported hematologic toxicity but was not clinically significant, with no unusual infections observed in the population, all of whom were followed up for at least 1 year. Fever, fatigue, and other symptoms that occurred during oblimersen infusions were manageable and did not affect administration of decarbazine at the recommended dose. Reported laboratory toxicities were similar to those observed with dacarbazine alone. Elevations in serum transaminase levels were transient with both the 14-day and 5-day oblimersen infusions and were not dose limiting.

Antitumor activity was reported in 6 of 14 patients, including 1 complete response, 2 partial responses, and 3 minor responses, with disease stabilization for at least 1 year and a median overall survival >17 months. The median survival in this small group of patients compares favorably with the 4–5-month overall survival time generally reported in patients with advanced melanoma who have failed first-line systemic chemotherapy (Middleton et al., 2000; Chapman et al., 1999; Eton et al., 1998; Falkson et al., 1998). One of these patients, a 90-year-old woman with bulky metastatic disease measuring >5 cm at baseline in pelvic lymph nodes and at the site of a previous skin graft, demonstrated an initial response after two cycles of decarbazine with oblimersen 6.5 mg/kg per day and a complete response after four treatment cycles (Jansen et al., 2000) (Fig. 9). In addition, a biopsy of the cutaneous area previously posi-

tive for melanoma demonstrated only fibrosis, indicating a pathologic complete response. A localized brain metastasis developed, which was successfully treated with gamma knife therapy, but there has been no other site of relapse. This patient's survival exceeded 2 years.

All patients demonstrated Bcl-2 protein expression in cutaneous melanoma metastases (measured by Western blot analysis) at baseline (Jansen et al., 2000). Based on serial tumor biopsies, decreases of 75% in Bcl-2 protein concentrations during oblimersen administration were observed in some patients (10 of 12 evaluable patients with plasma oblimersen concentrations >1 μg/ml) (Fig. 10). These results establish proof of principle in human subjects of downregulation of the target protein within tumors following systemic administration of an AS-ON.

Based on these results, a multicenter, randomized trial comparing the effectiveness of decarbazine with or without oblimersen in the treatment of chemotherapy naive patients with advanced unresectable stage III/IV malignant melanoma has been initiated.

Prostate cancer. PRECLINICAL EXPERIENCE. The transition from androgen-dependent to androgen-independent prostate cancer is often associated with aberrant high expression of Bcl-2, which confers resistance to the apoptotic actions of hormone therapy, chemotherapy, and radiation therapy (Gleave et al., 1999; Scott et al., 2001). High levels of Bcl-2 were linked with resistance and increased cell survival of LN prostate cancer cell sublines following radiation exposure (2–8 Gy) (Scott et al., 2001). Oblimersen has been shown to significantly inhibit cell growth of the prostate cancer cell line DU-145, a model of hormonally insensitive advanced prostate cancer (Campbell et al., 1998). *In vitro* incubation of Shionogi tumor cells, an androgen-dependent mouse tumor model, with mouse Bcl-2 antisense oligonucleotide plus docetaxel reduced cell viability by 50% and induced apoptosis (Gleave et al., 1999).

When mouse Bcl-2 AS-ON plus docetaxel was administered *in vivo* to mice bearing Shionogi tumors, the time to androgen-independent recurrence was significantly prolonged (Gleave et al., 1999). Combined treatment of mice bearing androgen-independent recurrent Shionogi tumors with mouse Bcl-2 AS-ON and micellar paclitaxel synergistically induced tumor regression and growth inhibition vs. either single agent (Gleave et al., 1999). In a similar study in androgen-dependent Shionogi tumor-bearing mice, combination treatment with mitoxantrone and mouse Bcl-2 AS-ON enhanced mitoxantrone cytotoxicity in a dose-dependent manner vs. controls (Tolcher et al., 1999). Downregulation of Bcl-2 by the AS-ON occurred in a dose-dependent, sequence-specific manner. These effects were also observed when docetaxel plus oblimersen were administered in an androgen-independent human prostate cancer xenograft (PC3) model, with no evidence of tumor regrowth in 3 of 10 mice and long-term (>60-day) survival in 4 of 10 mice (Table 3 and Fig. 6) (Tolcher et al., 2001).

CLINICAL EXPERIENCE. Oblimersen has undergone evaluation in clinical trials as a single agent (Morris et al., 1999) and in combination with weekly paclitaxel (Scher et al., 2000; Morris et al., 2002), mitoxantrone (Chi et al., 2001), or docetaxel (de Bono et al., 2001). In each of these trials, oblimersen was well tolerated, and antitumor responses were observed, supporting continued evaluation of oblimersen in androgen-independent prostate cancer. Evaluation of docetaxel combined with oblimersen is ongoing.

Breast cancer. PRECLINICAL EXPERIENCE. Preclinical studies established that estrogen withdrawal increases resistance to cytotoxic agents in Bcl-2-expressing breast cancer cell lines (Teixeira et al., 1995) and that estrogen withdrawal-induced breast cancer tumor regression in nude mice is prevented by Bcl-2 (Pratt et al., 1998). Exposure to oblimersen in MDA-MB-231 cells induced apoptosis, which was further enhanced by subsequent exposure to docetaxel or paclitaxel (D. Yang et al., 1999).

Incubation of breast cancer cell lines with oblimersen caused growth inhibition and reduced Bcl-2 protein in MCF-7 cells but not in tamoxifen-resistant LCC2 cells (Lilling et al., 2000). Transient Bcl-2 downregulation by oblimersen in MCF-7 and MDA435/LCC6 human breast cancer cells caused a >80% reduction of Bcl-2 protein levels in a sequence-specific manner for both lines (Chi et al., 2000). Combined treatment of oblimersen with cytotoxic agents resulted in additive cytotoxicity in both cell lines.

In human breast cancer cell line xenografts, oblimersen alone or in combination with six different chemotherapy drugs inhibited tumor formation in a dose-dependent manner (D. Yang et al., 1999) (Table 3). Synergistic and complete tumor regression was observed with the combination therapies. Combining oblimersen with PSC833 (a P-glycoprotein inhibitor) and liposomal doxorubicin synergistically suppressed the growth of drug-resistant human breast cancer xenografts in SCID mice compared with any of the three agents alone (Lopes de Menezes and Mayer, 2001; Lopes de Menezes et al., 2000).

CLINICAL EXPERIENCE. The preclinical experience with oblimersen prompted initiation of a phase I study evaluating oblimersen plus weekly docetaxel in patients with advanced solid tumors and documented Bcl-2 expression, including 5 patients with advanced breast cancer (Chen et al., 2000). Oblimersen was administered in escalating doses (1–4 mg/kg per day) as a continuous i.v. infusion over 21 days along with i.v. docetaxel 35 mg/m² administered weekly (days 8, 15, and 22). Cycles were repeated every 28 days. Overall, the combination was well tolerated, with grade 3 thrombocytopenia observed in 2 patients. No other grade 3 or 4 toxicities were reported (the dose-limiting toxicity was fatigue; grade 3 thrombocytopenia also was reported in 1 patient). Tumor responses were observed in 2 patients with breast cancer. The MTD was not reached, and the investigators concluded further evaluation in advanced breast cancer was warranted (Hayes, 2001). Pharmacokinetic analysis demonstrated that oblimersen doses of 3–4 mg/kg per day resulted in higher plasma concentrations than those previously reported to produce synergy with docetaxel *in vitro*. Subsequently, 9 additional patients received oblimersen 5, 7, or 9 mg/kg per day on days 1–5, 12–13, and 19–20, with docetaxel 35 mg/m² on days 6, 14, and 21 of a 28-day cycle. Using this schedule, the majority of toxicities were grade 1 or 2 in severity, with only 1 patient developing grade 3 thrombocytopenia. Overall, 2 patients demonstrated a partial response, and 4 experienced disease stabilization.

Colorectal cancer. PRECLINICAL EXPERIENCE. Bcl-2 expression occurs in 30%–94% of patients with colorectal cancer, correlating with a negative prognosis in Duke's C colorectal cancer and a multidrug-resistance phenotype in multiple cell lines, including resistance to camptothecins (H.-B. Yang et al., 1999; Ochoa et al., 2001a,b). In cellular and nude mouse xenograft studies, oblimersen has demonstrated activity alone and in com-

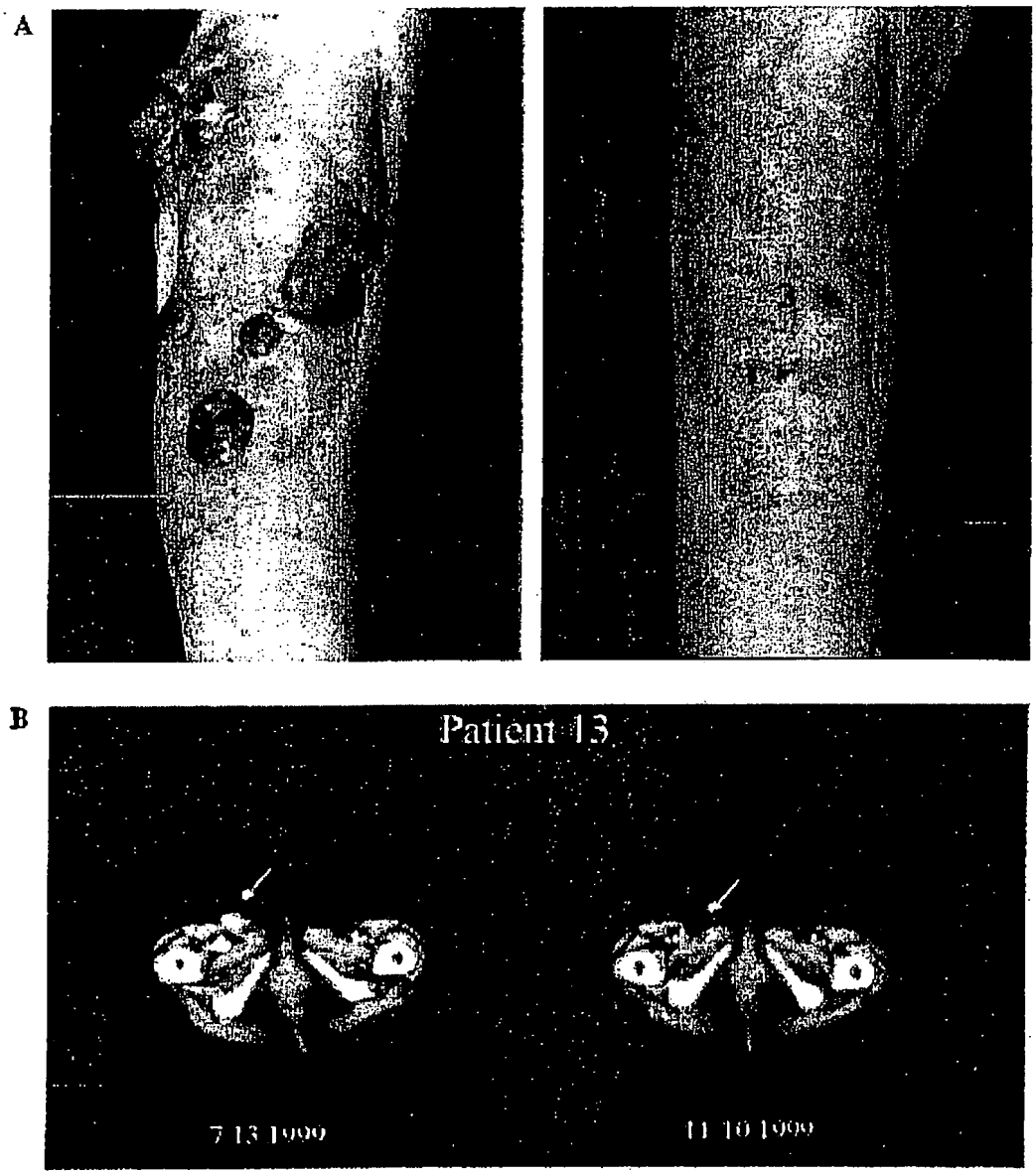

FIG. 9. Response following 4 weeks of treatment with oblimersen in a 90-year-old woman with malignant melanoma and bulky metastases to pelvic lymph nodes and at the site of a previous skin graft. (A) Skin metastases. (B) Computed tomography of pelvis. Reprinted with permission from Elsevier Science in Jansen, B., Wacheck, V., Heere-Ress, E., Schlagbauer-Wadl, H., Hoeller, C., Lucas, T., Hoermann, M., Hollenstein, U., Wolff, K., and Pehamberger, H. (2000). Chemosensitization of maligant melanoma by bcl2 antisense therapy. Lancet 356, 1728–1733.

bination with a protein kinase A AS-ON, causing significant prolongation of survival in tumor-bearing mice (Tortora et al., 2001) (Table 3).

CLINICAL EXPERIENCE. Oblimersen (3–7 mg/kg per day continuous i.v., days 1–8) has demonstrated activity in patients with metastatic colorectal cancer also receiving irinotecan (280–350 mg/m^2 i.v., day 6) (Ochoa et al., 2001a,b). Cycles were repeated every 21 days. Of the 17 patients enrolled, 1 patient (irinotecan-naive) achieved a partial response, and 3 patients (1 previously treated with irinotecan) achieved stable disease (Ochoa et al., 2001a). Toxicities, including grade 3/4 diarrhea, grade 3 nausea/vomiting, grade 4 neutropenia, and febrile neutropenia were dose limiting when oblimersen and irinotecan were administered at 5 mg/kg per day and 350 mg/m^2, respectively. Lower irinotecan doses were well tolerated, however, even with higher oblimersen doses.

Non-small cell lung cancer. PRECLINICAL EXPERIENCE. In a preclinical analysis, 3 NSCLC cell lines (NCI-H226 [squamous], NHCI-H228 [adenocarcinoma], NCI-H596 [adenosquamous]), which expressed both *bcl-2* and *bax* mRNAs with func-

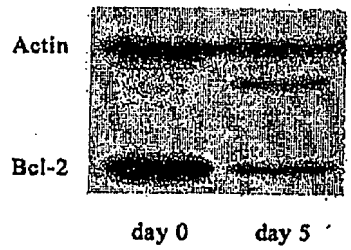

FIG. 10. Bcl-2 downregulation following 5 days of treatment with oblimersen in melanoma biopsy sample from a selected patient. The additional band shown on day 5 is consistent with the appearance of actin subunits associated with tumor cell apoptosis. Bcl-2 protein is 70% lower on day 5 by scanning densitometry, normalized against changes in the actin band. Reprinted by permission from Elsevier Science in Jansen, B., Wacheck, V., Heere-Ress, E., Schlagbauer-Wadl, H., Hoeller, C., Lucas, T., Hoermann, M., Hollenstein, U., Wolff, K., and Pehamberger, H. (2000). Chemosensitisation of malignant melanoma by bcl2 antisense therapy. Lancet 356, 1728–1733.

tional apoptotic pathways, were exposed to a Bcl-2 AS-ON, resulting in decreased Bcl-2 levels, reduced cell proliferation, decreased cell viability, and increased spontaneous apoptosis (Koty et al., 1999).

Vrignaud et al. (2002) recently studied the effects of oblimersen and docetaxel in a 3-arm dose-response study in mice, comparing the single agents with their combination (Table 3). Human lung carcinoma NCI-H460 was selected on the basis of its Bcl-2 expression level and chemosensitivity to docetaxel. Oblimersen was administered s.c. twice daily from day 8 to day 14 posttumor implantation in Swiss nude mice. Docetaxel was administered i.v. on days 9, 12, and 15. At the highest dose tested (8 mg/kg per dose, total dose of 112 mg/kg), oblimersen was active as a single agent with a 1 log cell kill. The highest nontoxic dose of docetaxel alone, 20.3 mg/kg per injection (total dose, 60.9 mg/kg), was also found to be active (2 log cell kill). Superior antitumor activity was obtained at the highest nontoxic combination (oblimersen 5 mg/kg per dose, docetaxel 20.8 mg/kg per injection), with a 3.1 log cell kill and complete tumor regression in 4 of 7 mice (Fig. 6). This oblimersen-docetaxel combination was well tolerated. The only side effect observed specific to the combination was a reversible edema occurring at the s.c. injection sits.

CLINICAL EXPERIENCE. Oblimersen is undergoing clinical evaluation in a randomized multicenter trial to compare response, response duration, time to progression, survival, and safety of docetaxel plus oblimersen vs. docetaxel alone in patients with stage IIIB/IV NSCLC who have previously received chemotherapy.

Small cell lung cancer. PRECLINICAL EXPERIENCE. Exposure of SCLC cell lines expressing high (NCI-H69), intermediate-high (SW2), and low (NCI-H82) basal levels of Bcl-2 to Bcl-2 AS-ONs plus etoposide, doxorubicin, or cisplatin (or all) resulted in synergistic cytotoxicity in all cell lines (Zangemeister-Wittke et al., 1998), supporting the potential value of Bcl-2 antisense therapy in patients with SCLC regardless of initial Bcl-2 protein levels.

CLINICAL EXPERIENCE. Rudin et al. (2001) conducted a phase I/II trial evaluating oblimersen (3 mg/kg per day, continuous i.v. on days 1–8) plus paclitaxel (175 mg/m^2, 3-hour i.v. infusion, day 6) in patients with chemorefractory SCLC. Of the 11 patients enrolled, 2 experienced stable disease, 1 of whom remained without evident progression for more than 6 months and who received 12 cycles of therapy. Hematologic dose-limiting toxicities occurred in 2 of the first 3 patients, resulting in paclitaxel dose reduction to 150 mg/m^2, and 1 patient developed a pruritic rash and was taken off study prior to receiving paclitaxel. These results suggest the combination may be tolerable and feasible in this refractory population. A clinical study to evaluate oblimersen plus carboplatin and etoposide in patients with untreated extensive-stage SCLC has been started.

Other tumor types. PRECLINICAL EXPERIENCE. Oblimersen has demonstrated activity (via induction of apoptosis and inhibition of tumor growth) either alone or in combination with other agents in a variety of human tumor xenografts (Table 3).

In human gastric cancer in a SCID mouse xenotransplantation model, oblimersen was administered alone (Wacheck et al., 2001a,b) or with cisplatin (Wacheck et al., 2001b). Combination with cisplatin significantly increased antitumor responses and increased survival compared with cisplatin or oblimersen alone without adding significant drug-related toxicity (Wacheck et al., 2001b).

Merkel cell carcinoma (an aggressive neuroendocrine skin tumor) implanted subcutaneously in SCID mice also demonstrated a significant reduction in tumor growth or complete ablation when the mice received oblimersen via continuous s.c. infusion (Schlagbauer-Wadl et al., 2000). In addition, investigators observed an enhancement of apoptosis, suggesting that oblimersen may improve treatment outcomes in this rare, aggressive tumor. Oblimersen also has demonstrated activity in bladder cancer cell lines (Duggan et al., 2001).

The activity of other Bcl-2 AS-ONs has been demonstrated in radiation-induced fibrosarcoma cells (Srivastava et al., 2001), in combination with photodynamic therapy against apoptosis-sensitive human epidermoid carcinoma (A431) cells (Srivastava et al., 2001), in bladder cancer cell lines (Duggan et al., 2001; Bilim et al., 2000), in a hepatocellular carcinoma rat xenograft model (Baba et al., 2000), and in a cholangiocarcinoma cell line (Harnois et al., 1997).

CHALLENGES OF Bcl-2 MEASUREMENT

Using techniques such as immunohistochemistry scoring, Western blotting, and PCR, many researchers have attempted to measure expression patterns and levels of Bcl-2 protein and mRNA. Four technical challenges may explain conflicting results. First, not only may individual patients variably express Bcl-2, but also tumor and normal cells in any sample may have differing levels of expression. Therefore, unless pure tumor cells are available, the interpretation of results may be clouded. Even in solid tumor material, there will be contaminating stromal and blood cells with variable levels of Bcl-2 expression. Second, tissue handling must maintain the validity of the measurements without degrading the nucleic acids or proteins. Third, the assay itself must be reproducible with appropriate controls. Fourth, baseline levels may not be reproducible because diurnal variation of Bcl-2 expression may further complicate the accuracy of any individual baseline measurement (Liu et al., 2001). These challenges have made reliable quantitative data difficult to obtain or interpret.

Even if Bcl-2 could be reproducibly and accurately measured in patient tissues, the absolute level of Bcl-2 may not be the critical factor. The relative levels of proapoptotic and antiapoptotic proteins, as well as the timing of expression (i.e., transient or constitutive), may be the decisive factors determining a cell's response to death factor stresses (e.g., cellular damage, cytotoxic chemotherapy) (Reed, 1999). The selectivity of Bcl-2 antisense for cancer cells over normal cells may be due to the relative inability of tumor cells to upregulate other antiapoptotic proteins in response to transient Bcl-2 downregulation. While acknowledging that aberrant Bcl-2 overexpression confers tumor cell resistance to many anticancer treatments, it appears that determining the specific Bcl-2 level in a patient with a given tumor is relatively unimportant. A small disruption in Bcl-2 protein levels may be all that is required to enhance the sensitivity of tumor cells. A decrease in Bcl-levels may shift the balance to provoke apoptosis or sensitize cells to apoptotic death irrespective of their baseline level of expression. Moreover, *in vitro* data suggest that tumor cells with somewhat lower levels of Bcl-2 expression are actually more sensitive to the synergistic cytotoxic effects of chemosensitization, possibly because of the greater magnitude of absolute reduction from basal levels (Konopleva et al., 2000). *In vitro* and clinical responses have been observed in both high and low Bcl-2-expressing cells and when the Bcl-2 protein level in tumor either decreases or increases (Gazitt et al., 2001; Marcucci et al., 2001).

One possible explanation for observing a rise in Bcl-2 protein levels or mRNA following treatment with oblimersen could be selective survival of high Bcl-2-expressing cells. Theoretically, if oblimersen is effective in triggering apoptotic cell death, only more resistant cells would remain after treatment. These resistant cells, although a minority population in the initial tumor cell denominator, become the new denominator and may have a relatively higher level of Bcl-2 expression. For example, if oblimersen were able to eliminate 90% of cells below a certain threshold level of Bcl-2 expression, the remaining 10% of cells could register higher Bcl-2 expression even though the tumor reduction was 1 log. This suggests that the dynamics of change in a dying tumor population treated with oblimersen may confound any analysis. Ultimately, the question of the predictive value of Bcl-2 baseline levels and change with antisense therapy can be answered meaningfully only in the clinic (with meticulous laboratory assessment). Further, these analyses may be moot if oblimersen is generally active in the treatment of cancer in combination with other therapies.

SUMMARY AND FUTURE PROSPECTS

With increased understanding of the role of apoptosis in neoplastic disease, extensive efforts are underway to exploit the pathways regulating this process as a therapeutic strategy. Bcl-2 expression appears to be an important mediator of clinically relevant chemotherapy resistance in a number of tumors, including NHL, AML, CML, CLL, MM, melanoma, prostate cancer, breast cancer, colorectal cancer, SCLC, and NSCLC. Oblimersen is able to specifically downregulate Bcl-2 levels *in vitro*. The ability of oblimersen to downregulate Bcl-2 within tumor tissue after systemic administration is well documented in mouse xenografts and a growing number of patient tumors sampled during therapy. There is preclinical evidence supporting a synergistic therapeutic role for oblimersen with cytotoxic agents in a wide spectrum of human cancers, including breast, lung, colon, prostate, gastric, Merkel cell, epidermoid, bladder, hepatoma, cholangiocarcinoma, lymphoma, MM, and acute and chronic leukemia. Clinical studies have provided evidence that oblimersen exhibits some activity when administered as a single agent and is especially effective when used in combination with traditional anticancer strategies. Randomized, multicenter clinical trials are currently underway to evaluate the efficacy and tolerability of oblimersen when administered with chemotherapy to patients with CLL, recurrent/refractory MM, malignant melanoma, and NSCLC. Potentiation of chemotherapy and other anticancer treatments with oblimersen Bcl-2 antisense therapy is a promising new apoptosis-modulating strategy, and results of ongoing trials should provide additional insight into this therapeutic approach.

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Address reprint requests to:
Stanley R. Frankel, M.D.
Director, Medical Operations
Genta Incorporated
2 Connell Drive
Berkeley Heights, NJ 07922

E-mail: Frankel@genta.com

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ATTACHMENT "C"

vitro and *in vivo*, thus supporting our continued development of a novel combination therapy of Taxol and *E1A* gene therapy and preparations for a phase II clinical trial.

#4811 Antisense *raf* oligodeoxyribonucleotide and tumor radiosensitization. Gokhale, P.C., McRee, D., Monia, B.P., Bagg, A., Rahmen, A., Dritschilo, A., and Kasid, U. *Departments of Radiation Medicine (P.C.G., D.M., A.D., U.K.), Pathology (A.B.), and Radiology (A.R), Georgetown University, Washington DC 20007; Isis Pharmaceuticals, Inc., Carlsbad, CA 92008 (B.P.M.) and NeoPharm, Inc., Bannockburn, IL 60015 (A.R.).*

The aim of the present study was to evalute the radiotherapeutic efficacy of a fully phosphorothioated and well characterized antisense *raf* oligodeoxyribonucleotide (ODN) (ISIS 5132/5132). Using our recently developed liposome encapsulation of ODN approach, we first compared the pharmacokinetic parameters of a liposomal formulation of 5132 (LE-5132) and 5132. The area under the plasma concentration-time-curve (AUC) was 5.8 times higher with LE-5132 as compared to 5132. Significantly higher ODN levels could be measured in most organs within 48 h of administration of LE-5132 compared with 5132 (liver, 18.4 fold; spleen, 31 fold; heart, 3 fold; lungs, 1.6 fold). In kidneys, the level was lower with LE-5132 (0.77 fold). Intravenous administration of LE-5132 into athymic mice bearing radioresistant human laryngeal squamous cell carcinoma (SQ-20B) inhibited Raf-1 expression in tumor tissue as compared to blank liposome-treated or untreated control groups. LE-5132 or ionizing radiation (IR) treatment caused transient inhibition of SQ-20B tumor growth. Remarkably, a combination of LE-5132 and IR treatments led to significant and sustained tumor regression for at least 27 days after the last treatment. Histopathological examination of tumor samples revealed a significant proportion of cells containing highly fragmented chromatin in LE-5132 + IR treatment group as compared to single agent and untreated groups. These *in vivo* data provide support for the use of antisense *raf* ODN in the management of radioresistant malignancies.

#4812 Membrane permeant peptide conjugates for imaging caspase-3 activity *in vivo*. Polyakov, V.R., Dahlheimer, J., Piwnica-Worms, D. *Washington University Medical School, St. Louis, MO 63110.*

Apoptotic stimuli converge on activation of effector caspases with evidence suggesting caspase-3/CPP32 and caspase-7 being central to the execution pathway of apoptosis in many different cell types. Quantification of the enzymatic activities of caspase-3 and caspase-7 *in vivo* could provide a means to monitor tumor cell commitment to apoptosis and directly guide therapeutic choices in patients. We describe prototypic membrane permeant peptide conjugates derived from TAT transduction sequences incorporating caspase-3/-7-specific substrate recognition sequences (DEVD) between TAT sequences and appropriate peptide-based motifs for chelating radionuclides such as Tc-99m (*a*-KGC), novel imaging agents have been engineered for analysis of caspase activity *in vivo*. TAT peptide conjugates were prepared by solid phase peptide synthesis, purified by RP-HPLC, and peptide identity confirmed by ESMS. Peptides were C-terminus labeled with fluorescein maleimide, or chelated with Re or Tc-99m by ligand exchange with gluccheptonate. TAT peptide imaging conjugates containing specific recognition motifs were cleaved by recombinant human caspase-3 *in vitro*. Upon incubation of Jurkat cells in buffer containing Tc-99m-TAT peptide, the complex rapidly ($t_{1/2}$ < 2 minutes) accumulated within cells, and showed equally rapid washout kinetics. Confocal microscopy revealed rapid cytoplasmic and focal nuclear accumulation of fluorescein derivatized conjugates. Furthermore, apoptosis was induced by pre-incubation of Jurkat cells for 5 hr in media containing C6-ceramide. Analysis of TAT peptide conjugates showed cleavage in ceramide-activated cells, but not in control cells, thereby trapping the C-terminus labeled chelate moiety within the cells. In this way, focal retention of radioactivity, a "hot spot", will be generated on scintigraphic images of tumors corresponding to enzymatically active caspase.

#4813 Induction of apoptosis in non-small cell lung cancer cells by p16INK4 is related to down-regulation of bcl-2 and Rb. Kataoka, M., Spitz, F.R., Schumacher, G.*, Liu, T-J.,* Fujiwara, T., Tanaka, T., Roth, J.A.*. Cristiano, R.J.* *First Dept. of Surgery, Okayama University School of Medicine, Japan. *Dept. of Thoracic and Cardiovascular Surgery, UT MD Anderson Cancer Center, Houston, TX 77030.*

We introduced a functional p16 cDNA into NSCLC cell lines expressing different combinations of normal and mutated p16, p53, and Rb genes via a recombinant adenovirus and analyzed the effect on cell growth. Adenovirially introduction of p16 protein in A549 cells (−p16/+p53) mediated apoptosis on five days after infection and inhibit the tumor growth in vitro and in vivo. Further analysis indicated that Bcl-2 and Rb expression was greatly reduced in A549 cells only on the fifth day after Adv/p16 infection. H1299 cells (−p16/−p53) infected with Adv/p16 also showed apoptosis only by an additional infection with an adenovirus expressing p53 which was accompanied by down-regulation of bcl-2 and Rb. Interestingly, Adv/Rb infection increase the expression of bcl-2 in Adv/p16 infected A549 cells. As a result, these studies suggest that p16 is capable of mediating apoptosis in the presence of wild-type p53, through a down-regulation of Rb protein followed by down-regulation of the anti-apoptotic protein bcl-2.

#4814 Tumor regression of human breast carcinomas by combination therapy of anti-bcl-2 antisense oligonucleotide and chemotherapeutic drugs. Yang, D., Ling, Y., Almazan, M., Guo, R., Murray, A., Brown, B., Lippman, M.E. *Lombardi Cancer Center, GUMC, Washington DC 20007 and Gente Incorporated, San Diego, CA 92121.*

Over-expression of BCL-2 protein is frequently found in cancer and may be an important negative regulator of apoptosis. An 18mer antisense oligonucleotide (G3139) designed to hybridize sequence in bcl-2 mRNA has been shown to inhibit bcl-2 expression in multiple cell lines with IC_{50} of 250nM. Antisense G3139 and controls (reverse or two-base mismatch) oligonucleotides were tested against two ER negative breast cancer cell lines (MDA-MB-231 and MDA-MB-435) and one ER positive cell line (MDA-MB-361) *in vitro* as well as tumor xenografts *in vivo*. All three cell lines express a high-level of BCL-2 protein. Treatment with G3139 in MDA-MB-231 cells induced apoptosis, inhibited cell growth and soft-agar colony formation. Following exposure to docetaxel or paclitaxel, markedly enhanced apoptosis and growth inhibition were observed in G3139 treated cells, but not in the control oligonucleotide treated cells. Treatment with G3139 alone can inhibit tumor formation *in vivo* 60% to 90% in MDA-MB-231, MDA-MB-435 or MDA-MB-361 xenografts in a dose dependent manner. Combination therapy of G3139 was carried out in animals at sub-optimal doses with six different chemotherapy drugs. Synergistic effects and complete tumor regression were observed only in the combined treatments of G3139 with docetaxel, paclitaxel or cisplatin. Mice remained tumor free for more than five months. No effects were observed with combined reverse or mismatch control oligonucleotides treatment. These data indicate that BCL-2 is an attractive target for anti-tumor therapy, and that bcl-2 antisense therapy should be investigated for clinical efficacy by itself and in combination withchemotherapeutic agents for breast cancer treatment.

#4815 Bcl-X_L, a new target for genomic cancer therapy: *In vitro* and *in vivo* downregulation and chemosensitization. Fennell, D.A., Corbo, M., Kuss, B., Monia, B.P., Dean, N.M., Cotter, F.E. *Department of Molecular Haematology, Institute of Child Health, 30 Guilford Street, London WC1 1EH, UK (D.A.F., M.C., B.K., F.E.C.) and Department of Molecular Pharmacology, ISIS Pharmaceuticals, 2280 Faraday Avenue, Carlsbad, CA 92008 (B.P.M., N.M.D.).*

BCL-X_L, an antiapoptosis protein, contributes to failure of chemotherapy in a number of malignancies. A second generation (mixed backone) antisense oligonucleotide, ISIS 16009 was investigated *in vitro* against leukaemia cell lines, SEMK2 with t(4;11) and BV173 with t(9;22) which express high levels of Bcl-X_L. In cell-free studies, ISIS 16009 induced RNaseH cleavage of Bcl-X_L RNA. Sequence and target specific reductions of intracellular Bcl-X_L RNA and protein were measured at 24 and 72 hours respectively. RNA catabolising potency (IC_{50}) was in the nanomolar range measured by northern blotting and concentration-response analysis. *In vitro*, treatment with VP16 produced Z-DEVD.fmk inhibitable (caspase dependent) phosphatidylserine (PS) expression which was enhanced after ISIS 16009, measured by cytofluorometric tolerance distribution analysis. Using *in-vivo* SCID/NOD SEMK-2 and BV-173 xenografts, 14 day subcutaneous infusions of 100 μg/day ISIS 16009 (or scambled control) were followed by 48 hours *ex-vivo* treatment with VP16 to observe effects on apoptosis tolerance. Bcl-X_L was reduced by 90%. Caspase (DEVD-ase) activity and PS expression were significantly increased. Conversely, 72 hour viability measured by MTT assay was lowest in the ISIS 16009 treated group. Bcl-X_L is a potential therapeutic target, and its downregulation may be of greatest benefit in the worse prognostic groups who fail to respond to chemotherapy due to its overexpression.

#4816 Antitumor effects elicited by antisense-mediated downregulation of the IGF-I receptor: From the bench to the bedside. Resnicoff, M., Andrews, D.W., Kenyon, L., Curtis, M., Merli, G., Baserga, R., Flanders, A., Iliakis, G., and Aiken, R.D. *Kimmel Cancer Center and Thomas Jefferson University Hospital, Philadelphia, PA 19107.*

Downregulation of the IGF-I receptor in C6 rat glioblastoma cells by antisense strategies results in massive apoptosis of tumor cells *in vivo*, leading to abrogation of tumorigenesis in nude mice. In addition to the apoptotic effect, antitumor responses are elicited in immunocompetent animals, protecting them from subsequent tumor challenge and causing regression of established tumors with no further recurrence. These findings in animal models have now been tested in 12 patients with malignant glioma, who had previously failed conventional therapies including radiation. Autologous glioma cells, harvested at craniotomy, were treated *ex vivo* with IGF-I receptor antisense oligodeoxynucleotides prior to subcutaneous reimplantation within diffusion chambers. This treatment was well tolerated and other than deep vein thrombosis noted in the first 4 patients, no other side effects were reported. Radiographic and clinical responses were observed in 60% of the patients, including 2 complete remissions at 14 and 6 months. The updated results from these studies will be presented and the possible mechanisms involved in these responses will be discussed.

#4817 Inhibition of epidermal growth factor receptor tyrosine kinase family members by the covalent antagonist CL-387,785. Discafani, C.M., Floyd, Jr. M.B., Johnson, B.D., Nilakantan, R., Nunes, M., Shen, R., Wang, Y.-F., Wissner, A., Greenberger, L.M. *Oncology and Immunoinflamatory Research, Chemical Sciences, and Computational Chemistry, Wyeth-Ayerst Research, Pearl River, NY 10965.*

ATTACHMENT "D"

NHL. Two patients had open lung biopsy which showed interstitial pneumonia. One patient died of ventilatory failure. The remaining 7 patients were treated with prednisone taper. Six of the treated patients had improvement between 1 and 14 days (median 4 days) while one patient gradually improved over 12-18 months. Follow-up CXR on 6 patients showed improvement or resolution of interstitial disease. Only one patient was re-treated with fludarabine and had return of symptoms with findings similar to his initial episode. Currently, we are comparing treatment factors such as preexisting pulmonary disease, and chest radiograph abnormalities to 150 patients treated at our institution who did not develop pulmonary toxicity to identify possible risk factors predicting toxicity. These data demonstrate that fludarabine, although generally safe and well tolerated, may uncommonly cause life threatening pulmonary toxicity. Clinicians should consider this diagnosis in patients treated with fludarabine who develop fever, hypoxia, and interstitial infiltrates without evidence of infection or disease progression. Fludarabine related pulmonary toxicity appears to respond to systemic corticosteroids. Given the recurrence of toxicity in one patient with retreatment, great caution should be taken before considering fludarabine rechallenge.

Abstract# 3273 Poster Board #-Session: 737-III

EVALUATION OF Bcl-2 ANTISENSE OLIGONUCLEOTIDE DRUGS IN MULTIPLE MYELOMA.

Niels van de Donk*,[1] Marloes Kamphuis*,[1] Mirjam van Dijk*,[2] Okke de Weerd*,[2] Henk Lokhorst,[2] Andries Bloem*.[1] *[1]Immunology, University Medical Center Utrecht, Utrecht, The Netherlands; [2]Hematology, University Medical Center Utrecht, Utrecht, The Netherlands.*

Multiple myeloma (MM) is characterized by the presence of a slowly growing plasma cell tumor mass, linked to the balance between proliferation and apoptosis. Bcl2 protein, commonly expressed in MM cells, has been shown to prevent apoptosis induced by myeloma therapies including dexamethasone, biologic, and cytotoxic drugs. An antisense oligonucleotide (ASO) targeting Bcl-2, G3139 (Genasense, Genta Inc.) has been shown to downregulate Bcl-2 and produce responses in patients with drug-resistant B-cell lymphoma (Waters, J., et al, J.C.O. 9:1812, 2000). Here, preclinical studies of Bcl-2 ASO evaluated potential applications for myeloma therapy. Incubation of human plasma cell lines with FITC-labeled ASO resulted in a time and dose dependent up-take in the cytoplasm and nucleus. The kinetics of ASO uptake differed between different plasma cell lines. Incubation with G3139, but not control sense or scrambled oligonucleotides, resulted in a time and dose dependent (up to 10-fold) decrease in Bcl-2 RNA, measured by RT-PCR. The same treatments led to > 75% reduction of Bcl-2 protein levels in the plasma cells after 4 days of exposure, without significant reduction in actin or Bax proteins, supporting a sequence-specific antisense mechanism. G3139-induced decreased Bcl-2 protein in U266 plasma cells enhanced apoptosis and cytotoxicity from doxorubicin. These results support the role of Bcl-2 in treatment-resistance of MM, and they suggest that Genasense, an agent already in Phase III trials in other indications, should also be evaluated for myeloma therapy.

Abstract# 3274 Poster Board #-Session: 738-III

IN VITRO AND *IN VIVO* EFFECTIVENESS OF ARSENIC TRIOXYDE IN A MURINE FORM OF T PROLYMPHOCYTIC LEUKEMIA (T-PLL).

Christian Rocher*,[1] Martine Chopin*,[1] Hervé Dombret*,[2] François Sigaux,[1] Marc-Henri Stern*.[1] *[1]U462, INSERM, IUH, Paris, France; [2]Hematologie Adultes, Hopital St Louis, Paris, France.*

T-cell prolymphocytic leukemia is a rare form of mature T-cell leukemia characterized by activation of the *MTCP1/TCL1* oncogene family. It is generally resistant to conventionnal chemotherapy and prognosis remains poor despite some advances achieved with purin analogs and anti-CD52 antibody. Absence of T-PLL cell line and rarity of the disease have interfered with progress in treatment. Transgenic mice for *MTCP1* (CD2-p13) develop a leukemia similar to human disease and could be used to test therapeutics. Because arsenic trioxyde (As_2O_3) has recently showed anti-leukemic effects in activated T lineage malignancies, we here evaluated its potential interest in T-PLL, by studying the *in vitro* and *in vivo* effects of As_2O_3 onto murine T-PLL.

Leukemic cells from transgenic mice CD2-p13 were used to generate cohorts of H2-compatible immunocompetent mice bearing T-cell leukemia with high counts of lymphocytes and large splenomegaly. Viable leukemic cells were obtained from tumoral spleens for *in vitro* studies. As_2O_3 reduced cell viability at clinically achievable concentration. Viability of leukemic cells exposed to 1µM As_2O_3 was 75±2% that of untreated cells at 24 hours and decrease to 47±3.9% at 48 hours. Cytotoxicity of As_2O_3 was similar to that of fludarabine at 1µM. As_2O_3 was synergized by 125µM ascorbic acid (Vit C) (cell viability: 14±5% at 24 hours, 0% at 48 hours) but not by 1mM buthionine sulfoxide (BSO), both agents interfering with ghrathione metabolism. By contrast, normal lymphocytes were less sensitive to As_2O_3 alone (viability: 85±1.2%) or in combination with Vit C (57±3%). To determine the *in vivo* effect of As_2O_3, four groups of five mice were treated intra peritoneally, 5 days a week for 4 weeks (20 injections) with placebo, As_2O_3 (5µg/g/d), Vit C (500µg/g/d), and As_2O_3+Vit C, respectively. Treatment was started 14 days after tumoral transplantation. No adverse effect was observed. Appearence of lymphocytosis and splenomegaly was delayed in the 2 groups with As_2O_3. Furthermore, survival of mice treated with As_2O_3 alone or in combination with Vit C was significantly prolonged (mean survival in days: 57.6±0.8 for As_2O_3; 58.6±1.2 for As_2O_3+Vit C versus 45±0 for placebo and 46±0 for Vit C) ($p<0.0001$).

This study demonstrates that As_2O_3 can induce clinical and biological responses and can improve survival in murine T-PLL. This is the first evidence to support clinical trials using As_2O_3 in human T-PLL.

Abstract# 3275 Poster Board #-Session: 739-III

COMBINATION FLUDARABINE, CYCLOPHOSPHAMIDE AND RITUXIMAB FOR PREVIOUSLY TREATED PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA (CLL).

Guillermo Garcia-Manero,[1] Susan O'Brien,[1] Jorge Cortes,[1] Francis Giles,[1] Stefan Faderl,[1] Susan Lerner*,[1] Maher Albitar*,[2] Hagop M. Kantarjian,[1] Michael J. Keating.[1] *[1]Department of Leukemia, University of Texas M.D. Anderson Cancer Center, Houston, TX, USA; [2]Department of Pathology, University of Texas M.D. Anderson Cancer Center, Houston, TX, USA.*

Treatment options for patients with relapsed CLL are limited. In this report, we present our preliminary results with a biochemotherapy combination using rituximab (a monoclonal antibody against CD20) with fludarabine and cyclophosphamide. During course 1, rituximab is given at a slow rate at 375 mg/m2 on day 1 followed by fludarabine 25 mg/m2 and cyclophosphamide 250 mg/m2 on days 2-4. During subsequent courses (2-6), rituximab is given at 500 mg/m2 on day 1 and fludarabine and cyclophosphamide are given at the same doses but on days 1-3. Of 84 patients registered, 43 are currently evaluable after >3 courses. Patient characteristics: median age 57 years (range: 18-74); 65.1% male; 44 % Rai Stage IV; median platelet count 110 (range: 15-367); median hemoglobin 12.2 (range: 6.8-16.0); median WBC 45.8 (range: 2.7-311); 3 or more lymph node sites involved 58%; B2-microglobulin greater than 3: 79%; 83 % performance of 1 or less. Of the 43 patients, 1 had SLVL and 1 marginal zone lymphoma. Median number of prior treatments 3: 9.3% of the patients had received alkylating agents only, 65.1% had been sensitive to fludarabine containing regimens, 20.9% had been resistant to fludarabine. Medium follow up is 5 months. Using NCI criteria, CR rate is 14% (all in the fludarabine sensitive group, 21%), nodular PR 14%, PR 42%, stabilization of disease 4.7%, no response 20.9%. Total response rate 69.9%. One patient died early. Five other patients have died, none during the first course, 3 of them due to progression of disease. Serious toxicities from the treatment include 8 episodes of pneumonia, 4 episodes of neutropenic fever, 2 of sepsis and 1 prolonged myelosuppression. Other frequent toxicities related to rituximab include fever, chills and nausea; hypotension occurred in 5 (12%) patients. In summary, fludarabine, cyclophosphamide and rituximab is a very active and well-tolerated program for patients with previously treated CLL, in particular for fludarabine sensitive disease. Studies evaluating the efficacy and toxicity of this program are ongoing.

Abstract# 3276 Poster Board #-Session: 740-III

DCEP (DEXAMETHASONE, CYCLOPHOSPHAMIDE, ETOPOSIDE AND CISPLATIN) + G-CSF IS AN EFFECTIVE REGIMEN FOR PERIPHERAL STEM CELL COLLECTION IN MULTIPLE MYELOMA.

M. Lazzarino,[1] A. Corso*,[1] L. Barbarano*,[1] E.P. Alessandrino*,[1] S. Pava*,[2] D. Ferrari*,[3] M. Fiumanò*,[2] G. Frigerio*,[2] L. Isa*,[2] A. Luraschi*,[2] S. Montanara*,[2] A. Nosari*,[1] D. Perego*,[2] G. Pinotti*,[3] F. Rodeghiero*,[1] A. Santagostino*,[2] M. Savarè*,[2] G. Ucci*,[2] L. Uziel*,[2] A. Vismara*,[2] E. Morra*.[1] *[1]The Divisions of Hematology of, IRCCS Policlinico S.Matteo University of Pavia, Osp. Niguarda Milano, Osp. S.Bortolo Vicenza; [2]The Internal Medicine - Hematology/Oncology of Abbiategrasso, Como Valduce, Desio, Gorgonzola, Lecco, Lagnano, Magenta, Milano S.Paolo, Omegna, Rho, Sondrio, Varese, Verbania, Vercelli, Italy.*

Peripheral blood stem cell (PBSC) transplantation following high-dose chemotherapy represents an integral part of front-line or salvage therapy in patients (pts) with multiple myeloma (MM). DCEP (dexamethasone 40 mg x 4 d and 4-day continuous infusion of daily doses of cyclophosphamide 400 mg/m², etoposide 40 mg/m², and cisplatin 10 mg/m² with subsequent G-CSF 300 µg s.c. until neutrophil recovery) has proved to be an effective salvage therapy for refractory-relapsed MM pts. Little is known, however, about its potential for mobilization of PBSC. We studied the efficacy of DCEP as mobilizing therapy in 29 MM pts. Of these, 21 had previously received chemotherapy with VAD only and 8 with alkylating agents. Characteristics of pts at time of DCEP were : 16 M/13 F; median age 53 yrs (30-68); 16 IgG, 7 IgA, 5 BJ, 1 nonsecretory. The median interval from first treatment was 6 mos (2-51). At start of DCEP, 18 pts showed a responsive disease while 11 were refractory to one o more lines of therapy. First leukapheresis was performed at peripheral blood CD34+ counts ≥20/µl. Mobilization was successful (minimum transplant dose $2x10^6$/kg CD34+ cells) in 25/29 pts (86%). The 4 patients who did not mobilize stem cells, all had been previously exposed to alkylating agents, 3 of them for a period longer than 6 months. The median time to first leukaferesis was 13 days (range 10-15). The median harvest of CD34+ cells was $5.75x10^6$/kg (range 2.1-22.4) with a median of 2 leukaphereses (range 1-4) per pt. The CD34+ cells harvest was $6.1x10^6$/kg in the 21 pts previously treated with VAD only. There was no treatment-related mortality. The side effects of DCEP were mild. No neutropenia <500/µl nor thrombocytopenia <50.000/µl were observed. One pt requested a transfusion of packed red cells. There was no need of hospitalization after therapy. In conclusion, DCEP in addition to its antitumor efficacy represents a safe, well tolerated and highly efficient mobilization regimen.

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